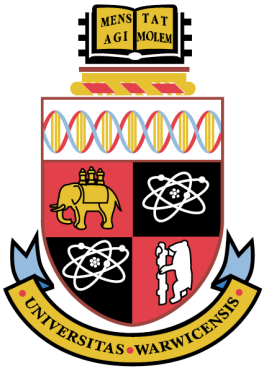

Nitrogen use efficiency in plants: how roots and nodules are balanced in *Medicago*

By

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Thesis

Submitted to the University of Warwick for the degree of

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has not been submitted for a degree at any other University. Except where specially stated, all of the work described in this thesis was carried out by the author in the University of Warwick, School of Life Sciences from October 2010 until September 2014.

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Publications

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Abstract

Nitrogen availability is central to global food security. Understanding how plants respond to nitrogen could help develop crops with improved nitrogen use. This study aims to investigate how lateral root (LR) and nodule development are balanced in response to low (0.1 mM) and high (5 mM) NH_4NO_3 concentrations in the model legume *Medicago truncatula* var. Jemalong A17 using phenotypic and gene regulatory network analysis.

High concentrations of NH_4NO_3 have an inhibitory effect on nodulation. The phenotypic measurements of root architecture of A17 in response to NH_4NO_3 and rhizobia showed that at high NH_4NO_3 rhizobia affected LR development. Total root size was less ($P < 0.05$) in rhizobia inoculated samples compared to the absence of rhizobia. This was due to a shorter primary root and less number of LRs. Whole genome profiling of early (2 and 6 hour) root responses to high NH_4NO_3 and rhizobia identified 4793 genes that were differentially expressed (significance cutoff of $P<0.05$). The early (2 and 6 h) responses to high NH_4NO_3 were mainly independent of the rhizobia effect and N-induction especially at 2 h time point was the predominant response. Some of the components of the autoregulation of nodulation (AON) pathway are also involved in LR development. The *sunn-1* mutant is impaired in correct AON signals that lead to hypernodulation with short LRs at low N. Whole genome expression analysis of A17 and *sunn-1* root samples identified 7186 significantly ($P<0.05$) expressed genes showing rhizobia and/or *SUNN*-regulated responses. These genes could be components of the AON pathway with putative role in balancing the number of nodules with LR development. Using motif analysis tools we identified motifs with putative promoters that were mainly Nodule specific cysteine rich peptides (NCRs). We suggest that these NCRs may be involved in regulating LR and nodule development depending on the plant N status.

List of abbreviations and symbols

-Rhiz	Mock inoculated
+Rhiz	Rhizobia inoculated
ABA	Abscisic acid
ACC	Ethylene precursor 1-aminocyclopropane carboxylic acid
AMT1	Ammonium transporter 1
ANR1	*Arabidopsis* nitrate regulated 1
AON	Autoregulation of nodulation
AVG	L-α-(2-AminoethoxyVinyl)Glycine hydrochloride
BAP	6-Benzylaminopurine, benzyl adenine
BEE1	Brassinosteroid enhanced expression 1
BEE2	Brassinosteroid enhanced expression 2
BEE3	Brassinosteroid enhanced expression 3
bHLH	Basic/helix-loop-helix
BR	Brassinosteroids
bZIP	Basic leucine zipper
C	Carbon
CCA1	Circadian clock-associated protein 1a
CCaMK	Calcium/calmodulin-dependent protein kinase
CRP	Cysteine rich peptides
dpi	Days post inoculation
dpt	Days post treatment
DTT	DL-Dithiothreitol
ENOD	Early nodulins
ERF	Ethylene responsive transcription factor
ERN	ERF required for nodulation
EtBr	Ethidium bromide
FC	Fold change
GA	Gibberellin
GRP	Glycine rich peptide
HAR	Hypernodulation Aberrant Root formation
HATS	High affinity transport systems
HY5	Elongated hypocotyl 5
HYH	HY5-Homolog
ICP	Inductively Coupled Plasma Atomic Emission Spectroscopy
JA	Jasmonic acid
JA	Jasmonate acid
LATS	Low affinity transport systems
LBD	Lateral organ boundary domain
LCO	Lipochitooligosaccharides
LR	Lateral root
LRP	Lateral root primordia
LRPi	Lateral root primordia stage i
LRPii	Lateral root primordia stage ii

LRR-RLK	Leucine-rich repeat receptor-like kinase
LSD	Least significant difference
LysM	Lysine Motif-Receptor-Like Kinase
MtDMI2	*Medicago truncatula* doesn't make infection 2
MtSUNN	*Medicago truncatula* super numeric nodules
N	Nitrogen, NH_4NO_3
NAA	1-Naphthaleneacetic acid
NARK	Nodule Autoregulation Receptor Kinase
NCR	Nodule Cysteine Rich secreted peptide
NF	Nod factor
NIN	Nodule Inception protein
NiR	Nitrite reductase
NLP7	NIN-like protein 7
NR (NIA)	Nitrate reductase
NSP2	Nodulation-signaling pathway 2 protein
P	Phosphorus
PCR	Polymerase chain reaction
PIN	Pin formed
PNR	Primary Nitrate Response
PR	Primary root
PSSM	Position-specific scoring matrix
pt	Post treatment
PTT	Phosphinothricin
r	Pearson correlation coefficient
RMA	Robust Multi-Array Averaging
rpm	Revolutions per minute
RSA	Root system architecture
SA	Salicylic acid
SPL9	Squamosa promoter binding protein-like 9
SUNN	Super numeric nodules
T101	Threonine 101
TCA	Tricarboxylic acid cycle
TF	Transcription factor
TML	Too much love
WT	Wild type

Chapter 1

Introduction

1.1 Nitrogen, a fundamental molecule for plants

Nitrogen (N) is an essential component of molecules including nucleic acids, amino acids and phytohormones thus it is essential for all aspects of plant development. N in its different forms such as nitrate and ammonium act as signal molecules regulating plant gene expression, metabolism, growth and development (Ruffel et al., 2008, Vidal and Gutiérrez, 2008, Bouguyon et al., 2012). N is available in the soil in several forms and it is acquired by plants as mineral (nitrate, nitrite and ammonium) and organic (amino acids and peptides) forms. Nitrate and ammonium are the two major forms of N available to plants in the soil.

N limitation is often a limiting factor for plant growth and development (Alvarez et al., 2012) and crop yield (Hirel et al., 2011). Soil N availability is heterogeneous, even in managed agricultural soils, and several factors cause fluctuations in the soil N content. Mineral N from added fertilizers or mineralized organic matter can be depleted through leaching or be released as N gases to the atmosphere (Hirel et al., 2011). The uptake of nitrate and ammonium by the roots makes the rhizosphere alkalised or acidic, thereby altering the soil N availability for plants (Marschner, 1995).

Atmospheric N_2 that comprises 80% of the atmosphere (Sanhueza, 1982) is not directly usable by plants. Atmospheric N_2 can enter the biological cycle by conversion into nitrate by lightning or photochemical conversion and into ammonia by the Haber-Bosch industrial fixation (Marschner, 1995). Legumes have also evolved this ability to use atmospheric N_2 in the form of

ammonium through the symbiotic relationship they establish with specific N_2 fixing soil bacteria.

N in the form of nitrate is taken up via plasma membrane-localised transporters. It is then assimilated into the primary organic forms of N glutamine and glutamate and then all other N-containing metabolites and macromolecules through a series of enzymatic reactions. The assimilatory reduction of nitrate takes place in two steps (Figure 1-1). Nitrate is reduced to nitrite (NO_2^-) by nitrate reductases (NR) in the cytosol in a two-electron reaction and then to ammonium (NH_4^+) by nitrite reductase enzymes (NiR). Ammonium is then assimilated into amino acids by the combined action of glutamine synthetase and glutamate synthase in plastids and chloroplasts (Crawford, 1995, Orea et al., 2001, Márquez et al., 2005, Bouguyon et al., 2012).

Several abiotic and biotic factors can affect the efficiency of the plant root in acquiring N (Schiefelbein and Benfey, 1991, Robinson, 1994). These include the availability of other nutrients or pathogen attack. To respond to external N availability and whole plant N status, and integrate other abiotic and biotic factors there are a number of plant regulatory responses. These include alterations to root system architecture (RSA) (Dastidar et al., 2011), regulation of uptake systems for nitrate and ammonium with different affinities (Bouguyon et al., 2012) and integration of local and systemic signaling pathways (Ruffel et al., 2008, Alvarez et al., 2012) that are activated in response to N.

1.1.1 N uptake systems

N availability to plants in the soil is heterogeneous, with plants facing simultaneous local high or low concentrations of N across the root system. Plants have evolved several uptake and transport systems to cope with their rapidly changing environment (Gutiérrez, 2012, Alvarez et al., 2012, Canales et al., 2014). Nitrate (Crawford and Glass, 1998) and ammonium (Ludewig et al., 2007) are acquired through two different uptake systems

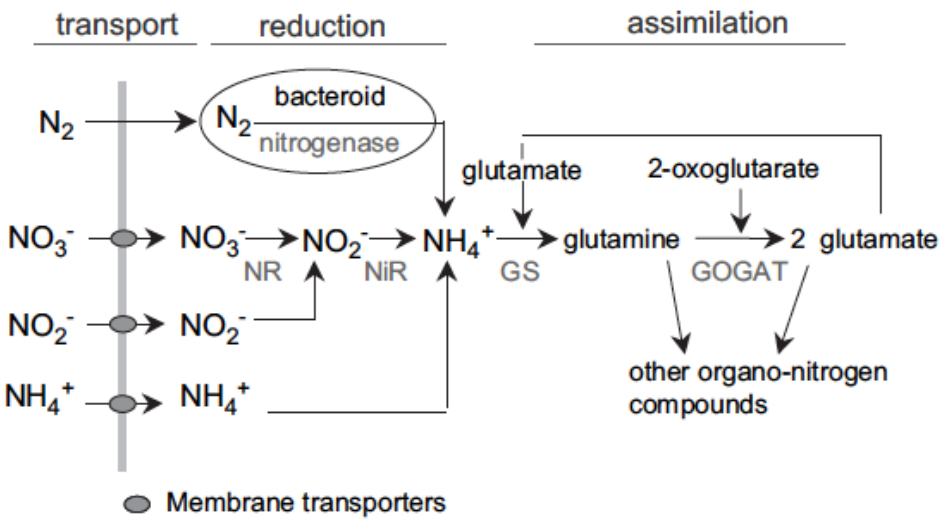


Figure 1-1: The process of nitrate assimilation (Márquez et al., 2005).

(Glass et al., 2002). Low affinity transport systems (LATS) that act at high N concentrations (>1 mM) and high affinity transport systems (HATS) that are activated at low (µM concentration ranges) N (Kraiser et al., 2011).

Studies in Arabidopsis have revealed that two nitrate transporter families known as NRT1 and NRT2 are responsible for transport of nitrate. NRT1 and NRT2 are symporters, transferring nitrate with H^+ in a symport mechanism and based on the pH gradient cross the membrane (Kraiser et al., 2011). *NRT2* encodes high affinity nitrate transporters (Bouguyon et al., 2012). *NRT1* encodes low affinity nitrate transporters with the exception of NRT1.1, which is a dual affinity nitrate transporter involved in both low and high affinity nitrate transport (Wang et al., 1998, Liu et al., 1999, Liu and Tsay, 2003). Ammonium is transported by members of the ammonium transporter 1 (AMT1) family including AMT1.1, 1.2, 1.3 and 1.5 (Alvarez et al., 2012). These proteins are either ammonium uniporters transferring ammonium along the electrochemical gradient or they are NH_3/H^+ co-transporters (Kraiser et al., 2011).

The ability of AtNRT1.1 to switch from low to high affinity modes is a regulatory mechanism enabling plants to respond rapidly to the changes in N availability. This regulation is via phosphorylation of the threonine residue 101 (T101). AtNRT1.1 acts as a high affinity nitrate transporter when it is phosphorylated (at low nitrate, <1 mM) and as a low affinity transporter when dephosphorylated (Liu and Tsay, 2003). AtCIPK23 is a signaling kinase that phosphorylates T101 of AtNRT1.1 at low nitrate concentration leading to a low level primary high affinity response and a weak induction of AtNRT2.1 (Ho et al., 2009). At high nitrate concentration (> 1mM), T101 is not phosphorylated, thus AtNRT1.1 functions as a low affinity transporter/sensor and the expression of *AtNRT2.1* is highly induced (Ho et al., 2009).

1.1.2 Signaling pathways responding to N availability and affecting RSA

N uptake systems are under the control of local and systemic signaling. The activity of the uptakes systems are regulated in a way that the net intake

of N depends on the plant need and demand (i.e. plant's current growth and developmental stage) rather than the N availability in the root environment (Imsande and Touraine, 1994). Roots respond to localized supplies of nitrate by a transient and localized increase in nitrate uptake activity and an increase in LR proliferation in the part of the root subjected to high nitrate (Forde, 2002). Shoot to root signals are involved in the systemic signals regulating the N response.

1.1.2.1 Regulation and signaling of N responses

In the primary nitrate response (PNR), nitrate rapidly induces the expression of a series of nitrate-related genes (Figure 1-2). The expression of a calcineurin B-like (CBL)-interacting protein kinase (CIPK8) is rapidly induced by nitrate and it positively regulates the expression of PNR genes such as nitrate transporter genes and genes involved in nitrate assimilation (Hu et al., 2009). Studies analysing the kinetic level of the induction of these genes by nitrate suggest that there are two high and low affinity response phases (Figure 1-2). Studies on *cipk8* mutant defective in the low affinity phase suggest that CIPK8 is involved in the low affinity phase and that these two phases are genetically distinct.

The dual affinity nitrate transporter NRT1.1 and the high affinity nitrate transporter NRT2.1 have dual functions as transporters and also as sensors in the roots for nitrate availability. They act together in the nitrate-signaling pathway (Figure 1-2) and this role is separate from their role as nitrate transporters. In *NRT1.1* mutants of *Arabidopsis* nitrate responsive genes are not regulated in response to nitrate. Suggesting the dual function of NRT1.1 as a nitrate transporter and sensor (Wang et al., 2009), mutants of *chl1* and *atnrt1.2* show altered root architecture even in the absence of nitrate, which could be an evidence for the roles of NRT1.1 and NRT2.1 as sensors and signal transducers (Guo et al., 2001, Little et al., 2005, Walch‐Liu and Forde, 2008). In the *chl1-9* mutant, proline residue 492 is changed to leucine by a point mutation. In this mutant, AtNRT1.1 nitrate uptake function is reduced at

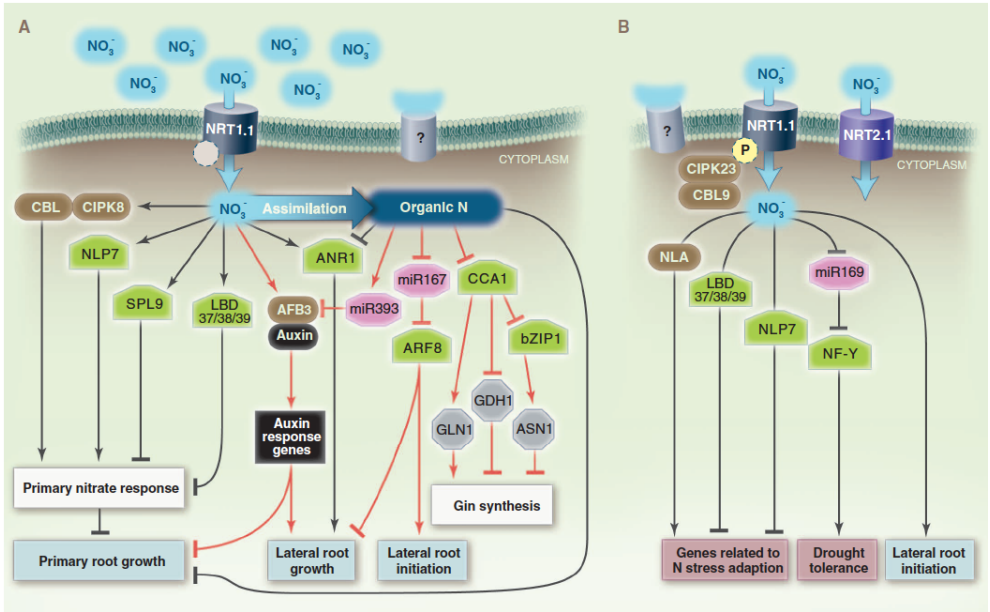


Figure 1-2: Known key molecular players of the signaling pathways regulating N responses (Gutiérrez, 2012). Several metabolic, physiological and developmental pathways are involved in the adaptive responses to N availability. Primary and long-term responses are activated in response to nitrate. The primary nitrate response signaling pathways comprises of (A) the kinase CIPK8-dependent (low-affinity: high nitrate concentration) and (B) CIPK8-independent (high-affinity: low nitrate concentration) pathways (Ho et al., 2009). NRT1.1 is a dual affinity nitrate transporter acting as a nitrate sensor and transporter. It is involved in the short-term induction and long-term repression of the genes involved in the nitrate transport and assimilation. The affinity of NRT1.1 is regulated by the kinase CIPK23 that is responsible for the phosphorylation of the T101 residue. NRT1.1 is dephosphorylated at high nitrate condition (> 1 mM) and functions as a low affinity transporter (A) and phosphorylated at low nitrate concentration (< 1 mM) to act as a high affinity transporter (B). The high affinity nitrate transporter NRT2.1 is a key component in the systemic signal regulating root nitrate uptake (Ruffel et al., 2008). It also has a separate role as a nitrate sensor suppressing LR initiation at low nitrate availability (Little et al., 2005). ANR1 controlling LR elongation in response to patches of nitrate supply and acts downstream NRT1.1 (Zhang and Forde, 1998). LBD 37/38/39, NLP7, HY5, HYH and SPL9 are other TFs participating in the nitrate signaling pathways. Green shapes: TFs, gray octagons: enzymes, pink octagons: microRNAs, and brown shapes: other regulatory molecules. Black lines: relationships obtained by molecular genetic approaches, and red lines: relationships discovered by systems biology approaches (Gutiérrez, 2012).

the low and high affinity range but the primary nitrate response of AtNRT2.1 is not affected by this change in amino acid (Ho et al., 2009). This could therefore suggest that the role of AtNRT1.1 as a nitrate transporter is independent of its role as a nitrate sensor.

In *Arabidopsis*, NRT2.1 is under the control of nitrate availability and N metabolites such as glutamate. Local supply of nitrate induces the expression of *NRT2.1* (Zhuo et al., 1999) while it is suppressed by the systemic signals from shoot to root when the plant N status is high (Gansel et al., 2001). AtNRT2.1 is one of the key components of the systemic signaling of nitrate response. When high nitrate concentration is supplied for several days it is downregulated by AtNRT1.1 (Muños et al., 2004, Krouk et al., 2006). AtNRT1.1 has the ability to sense the broad range of nitrate concentrations in the soil by its dual-affinity binding and phosphorylation/dephosphorylation abilities (Ho et al., 2009). In split root experiments when supplying one part of the root with high nitrate and subjecting the other part to nitrate starvation the expression of AtNRT2.1 increased in the nitrate fed section of the root in response to the N starved part. This suggests that AtNRT2.1 expression is under the control of shoot to root systemic signaling of N demand. The increase in expression level in the nitrate fed part of the root suggests that AtNRT2.1 is one of the first targets of the long-distance signaling that informs the roots of the whole plant's N status (Gansel et al., 2001).

One of the main regulators of the nitrate signaling pathway is the NIN-like protein 7 (NLP7) TF, a homologue of NIN (Nodule Inception) protein, which is one of the regulators of nodulation in legumes (Schauser et al., 2005). NLP7 is involved in the direct regulation of several steps of PNR and signaling pathways (Castaings et al., 2009, Marchive et al., 2013). Hundreds of regulatory and structural genes involved in nitrate metabolism and signaling have been identified as direct NLP7 targets showing early (within just 10 min) nitrate response (Marchive et al., 2013). The transcript abundance of three members of the lateral organ boundary domain (LBD) gene family, LBD37/38/39, is induced by nitrate and represses the biosynthesis of

anthocyanin and many N responsive genes including genes required for nitrate uptake and assimilation (Rubin et al., 2009). Other TFs that are key regulators of PNR are elongated hypocotyl 5 (HY5), HY5-homolog (HYH) and squamosal promoter binding protein-like 9 (SPL9). HY5 and HYH are bZIP TFs that positively regulate nitrate reductase (NIA) 2 and negatively regulate NRT1.1 in *Arabidopsis* (Jonassen et al., 2009). SPL9 is a negative regulator of NIR and NIA2 (Krouk et al., 2010b) (Figure 1-2).

1.1.2.2 Regulation and signaling of RSA development in response to N

The uptake rate of nitrate sensed and regulated by AtNRT2.1, rather than the external level of nitrate is the key factor causing changes in RSA. AtNRT2.1 acts as a nitrate sensor or signal transducer to regulated LR development in response to N availability (Little et al., 2005, Remans et al., 2006b). In *Arabidopsis NRT2.1* suppresses LR initiation at low nitrate and this suppression can be eliminated in the WT by increasing the concentration of external nitrate concentration. This repression is also released in the *Atlin1* mutant that carries a missense mutation in the *NRT2.1* gene (Little et al., 2005). Thus, NRT2.1 is involved on RSA changes in response to N availability: as a transporter it is involved in sensing and uptake of nitrate based on the external availability of nitrate and the N demands of the plant, with its signaling role triggering LR development in response to limiting N levels.

NRT1.1 that is expressed in the apex of the primary root (PR), apex and base of lateral root (LR), young emergent LR and LR primordia (Guo et al., 2001, Remans et al., 2006a) is also involved in the N-regulated changes of RSA. Studies on plants with mutation in NRT1.1 suggest that at low nitrate concentrations NRT1.1 is involved in LR primordia maturation and elongation and the first stages of PR growth at different concentrations of nitrate (Guo et al., 2001).

The MADS box transcription factor (TF) *Arabidopsis* nitrateregulated1 (ANR1) is one of the components of a signaling pathway affecting LR growth

rate in response to localized nitrate supply by controlling LR elongation in response to nitrate. In *Arabidopsis* transgenic plants that showed repressed levels of *ANR1* expression, localized nitrate supply did not lead to LR elongation (Zhang and Forde, 1998). NRT1.1 acts upstream of ANR1 in this signaling pathway controlling LR growth in response to external nitrate supply (Remans et al., 2006a) (Figure 1-2).

In *Arabidopsis thaliana* LR initiation and higher order LR branching is increased by localized supply of ammonium but not LR elongation (Lima et al., 2010). The role of AtAMT1.3 in regulating LR branching in response to ammonium is independent of ammonium transport and this suggests that this transporter also has a signaling role (Lima et al., 2010). Studies show that in *M. truncatula* both root nitrate and ammonium uptake are under the control of the systemic signallings of N status of the whole plant (Ruffel et al., 2008).

1.2 Dynamic regulation of LR and nodule development enables root architecture plasticity

Legumes are important food and biofuel crops. The advantage legumes have acquired is the symbiotic relationship between the root and the N-fixing soil bacterium that enables them to take up otherwise unusable atmospheric N (80% of the atmosphere). This process is termed as nodulation. Nodulation is, with a few exceptions, limited to leguminous species (Sprent, 2007). Genetic evidence shows that nodule development integrates pre-existing plant regulatory pathways that are related to LR organogenesis (Mathesius, 2003). The regulation of LR development itself shares parallels to root-mutualist interactions, although rather than microbes, plant pathways are regulated by environmental conditions including N and phosphorus form and availability.

LRs and nodules are both root lateral organs formed post embryonically by the reactivation of cell division in differentiated root cells in specific cell files (Malamy and Benfey, 1997, Stougaard, 2000) and are controlled spatially and quantitatively by hormone gradients (De Smet et al., 2007, Tirichine et al.,

2007, van Noorden et al., 2007, De Smet et al., 2008). The ability to form these new organs post embryonically gives plants potential plasticity to adapt to their environmental constraints. The formation of LRs is regulated principally by the availability of nutrients and this is controlled by hormone signaling pathways (López-Bucio et al., 2003). LR development and nodulation share a common environmental regulation. They are both induced in conditions of low N (rhizobia-dependent), and the development of the two organs is fundamentally linked. High N can inhibit the formation of new nodules (Streeter, 1985, Thimm et al., 2004) and influence N fixation capacity of existing nodules (Cabeza et al., 2014). LR development is regulated through local and systemic responses to N (Desnos, 2008). There is also evidence that symbiotic organisms can affect LR development (Maillet et al., 2011, Oláh et al., 2005).

LRs have existed for over 400 million years (Raven and Edwards, 2001). Nodules however, have evolved recently, around 60 million years ago possibly due to an environment that lacked N and was rich in CO_2 (Sprent, 2007). Thus, mechanisms that regulate nodule development may have been co-opted from the existing processes that regulated LR formation (Hirsch et al., 1997). Studies show that LRs and nodules share some similar developmental pathways. For example LR growth is slower in *Lotus japonicus* plants inoculated with rhizobia compared to control and the *har1-1* (*hypernodulation aberrant root formation*) mutant shows an even stronger phenotype. *Lotus brush* and *crinkle* mutants have reduced nodulation and are impaired in root development (Desbrosses and Stougaard, 2011). There are also mutants that form nodules similar to LRs. Peripheral vasculature are a characteristic of legume nodules. *M. truncatula lin-4* mutant forms vascular bundles located centrally in the nodules. These vascular bundled are similar to LRs (Guan et al., 2013). *M. truncatula nodule root (NOOT)* and its orthologue in *Pisum sativum cochleata (COCH)* are essential for the maintenance of the nodule identity through the nodulation process. Studies on *noot* and *coch* mutants gives some more evidence for the root evolutionary origin of nodule vascular bundles suggesting that *NOOT* and *COCH* may

have been used to repress root identity in the legume nodules (Couzigou et al., 2013).

Several known and well-characterized regulators of nodulation (Oldroyd, 2013) also affect LR development. For example, Nod factors are rhizobial-signaling molecules that have a crucial role in the rhizobia-legume cross talk. LR formation can also be stimulated by Nod factors and the genes involved (e.g. NFP, DMI1, DMI2, DMI3 and NSP1) are the same genes required for the Nod factor induced symbiotic response (Oláh et al., 2005). RNAi knockdown of the cytokinin receptor *MtCRE1* in *Medicago* results in reduction in nodule number and increase in LR number (Gonzalez-Rizzo et al., 2006). *latd* (*lateral root organ-defective*) mutants in *Medicago* are unable to form active nodules or complete LR formation with consequential effects on PR development (Bright et al., 2005). Together these developmental and molecular connections support the accepted hypothesis that the nodule structure arose from a LR blueprint (discussed in Mathesius, 2003) whilst also 'co-opting' or utilizing genetic interactions involved in AM interactions. (Deak and Malamy, 2005)

1.2.1 LR development

The tap root system in *Arabidopsis* consists of a PR formed during embryogenesis and LRs that are formed post-embryonically (De Smet et al., 2010). LRs are initiated close to the root tip and the fully developed LR emerges from the differentiation zone. LRs originate from the reactivation of cell division in differentiated pericycle cells that are located at the most outer layer of the vascular bundle (Tian et al., 2014). Recent studies on LR development in *M. truncatula* using DR5:GUS and DR5:VENUS-N7 reporter lines shows that endodermis and inner cortex also contribute in lateral root primordia (LRP) formation (Herrbach et al., 2013). DR5 marks local auxin accumulation and since LR initiation is triggered by auxin accumulation this promoter was used to study early stages of LR development. The DR5 activity at the early stage of LRP initiation is not only observed in pericycle dividing

cells but is also extended to epidermis and inner cortex (Herrbach et al., 2013). These are interesting differences in LR ontogeny with *Arabidopsis thaliana*. DR5:GUS expression profiling indicated that the endodermal and cortical cell divisions could be due to auxin accumulation (Herrbach et al., 2013).

Changes in auxin movement, perception of gravity and mechanical stimuli contribute to the regular distribution of LRs (Dastidar et al., 2011). Local auxin fluxes in root meristem are necessary for pericycle cells acquiring the identity of LR founder cell (De Smet et al., 2007, Dubrovsky et al., 2008). LR initiation takes place in a narrow developmental window. Studies on *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato) shows that distinct zones on the root where auxin content and response are minimal define the position of this developmental window for LR initiation (Dubrovsky et al., 2011). PIN3 and PIN7 are auxin efflux carriers from the PIN-Formed (PIN) family. They maintain the proximal auxin gradient with in the root and are essential for the correct positioning of the developmental window for LRs. In the zone of auxin minimum, pericycle cells have the highest probability of acquiring the identity of LR founder cells (Figure 1-3 a) (Dubrovsky et al., 2011). This 'priming' of pericycle founder cells (specification of these cells to divide and form a LR) that are located opposite the xylem poles occurs in a zone named the basal meristem (between the apical meristem and the elongation zone from the root tip) (Dastidar et al., 2011). Following the specification of LR founder cells and by the development of the PR, these primed pericycle cells are displaced further away from the root meristem. Cell division and initiation of development of the LRP is then triggered by a second auxin flux from the shoot (Dubrovsky et al., 2008). Auxin-induced expression of the auxin efflux carriers, encoded by the PIN genes (Figure 1-3 b) creates this auxin maxima leading to the first asymmetric cell division and cell fate respecification to acquire LRP identity (Laplaze et al., 2007). LR initiation starts with the asymmetric division of pericycle cells and occurs in the root differentiation zone (Dubrovsky et al., 2011). It is then followed by several

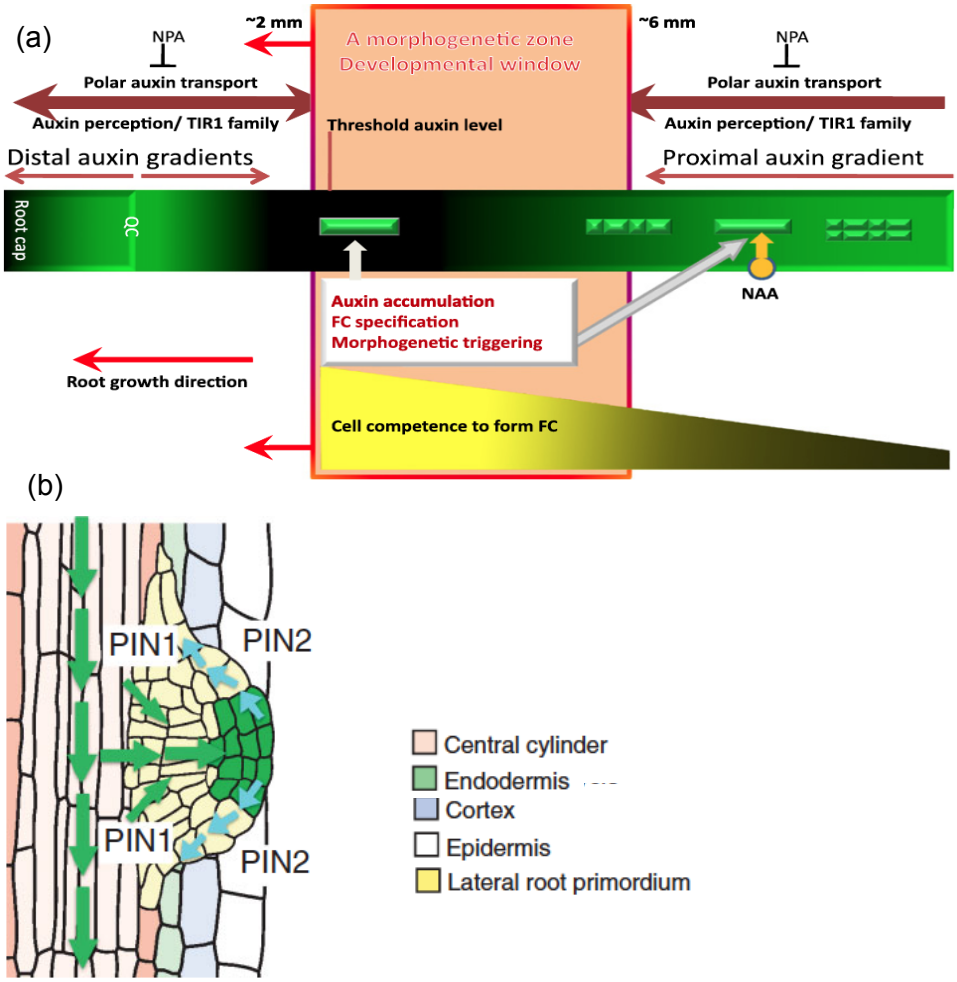


Figure 1-3: Auxin-mediated control of LR growth in *Arabidopsis*. a) A well-defined zone with a minimum auxin content and response along the root defines the positioning of a developmental window for founder cell specification and LR initiation in *Arabidopsis* and tomato (Dubrovsky et al., 2011). In an intact growing root two distal and one proximal auxin gradients separated by an auxin minimum zone are established that define a morphogenetic zone for LR initiation. At the zone with the minimum auxin concentration pericycle cells have the highest probability of acquiring the identity of founder cells (FC). In *Arabidopsis* roots the distal gradient usually ends at 0.2 mm from the root tip and the proximal auxin gradient starts 4.5-6 mm from the quiescent centre. This morphogenetic zone is dynamic and moves in the same direction and at the same rate as the root growth. NPA-sensitive polar auxin transport and auxin receptors of the TIR1/AFB family are essential for the function of this developmental window. Upon the application of external auxin (NAA) the distribution of the morphogenetic zone is rearranged activating the cells outside this zone to act as FCs (Dubrovsky et al., 2011). b) An auxin maxima leads to the first asymmetric cell division and cell fate respecification to acquire LRP identity. This second auxin flux is transferred from the shoot to the PR tip and LRP by PIN1-dependant auxin transport (green arrows) and is accumulated at the tip of the LRP forming an auxin maximum (green cells). Part of the auxin is then retrieved through a PIN2-dependent auxin route (blue arrows) (Dastidar et al., 2011).

rounds of anticlinal and periclinal divisions (Figure 2-4) to form a dome shaped LRP (Malamy and Benfey, 1997, Herrbach et al., 2013, Lucas et al., 2013).

The new LRP must emerge through overlaying endodermis, cortex and epidermis cell layers. Cell separation of the adjacent cell tissues makes it possible for the LRP to emerge and auxin has a critical role in this. Auxin, produced in the new LRP is transported to the cortical cells overlaying the new LRP where it acts as an inductive signal inducing the expression of PIN3, which creates an auxin flux towards the epidermis (Péret et al., 2013). The intracellular concentration of auxin however, is high enough for induction of the auxin influx carrier LAX3. LAX3 is expressed only in two files of cortical cells overlaying the new LRP. The consecutive expression of PIN3 and LAX3 prevents the expression of LAX3 in multiple cell files (Péret et al., 2013). LAX3 is required for emergence of the LRP through cortex and epidermis by controlling the auxin dependent induction of cell wall degrading enzymes (Swarup et al., 2008, Péret et al., 2013).

1.2.1.1 Hormonal control of LR development

Plant hormones and in particular auxin, cytokinin, ethylene, gibberellins (GA) and brassinosteroids (BR) have a central role in regulating LR growth. As previously mentioned, auxin influences all aspects of LR development such as initiation, positioning and patterning of LRP (De Smet et al., 2007, Dubrovsky et al., 2011) as well as emergence of a fully developed LR (Swarup et al., 2008, Péret et al., 2013). Studies show that plants impaired in auxin signaling form fewer LRs, and treatment of plants with auxin leads to an increase in the number of LRs (Laskowski, 2013). In *Arabidopsis* transgenic plants with reduced cytokinin concentrations, there was an increase in the number of LRs, suggesting that cytokinin is a negative regulator of LR development (Werner et al., 2003). Cytokinin regulates LR development by influencing the asymmetric cell division of the two adjacent founder cells. Downregulation of the expression of PIN genes by cytokinin inhibits the

formation of the auxin gradient required for this asymmetric cell division and LRP patterning (De Smet et al., 2007).

Cross talk between ethylene and auxin also regulates LR development due to ethylene interaction with auxin transport and perception. Ethylene inhibits LR formation at the early stages of initiation. In *Arabidopsis* and tomato, LR formation was reduced after treatment with ethylene or the ethylene precursor 1-aminocyclopropane carboxylic acid (ACC) compared to non treatment (Negi et al., 2008, Negi et al., 2010). Both *Arabidopsis* mutants in *ethylene resistant 1* (*etr1* or *ein1*), which confers a dominant negative ethylene receptor mutation, and *ethylene insensitive 2* (*ein2*), have an increased number of LRs (Negi et al., 2008). Enhancing ethylene synthesis by application of low concentrations of ACC induces LRP initiation. Treatment with higher levels of ACC affects LRP development by inhibiting the ability of pericycle cells to initiate new LRP and promoting the emergence of already formed LRP . Studies on *Arabidopsis* root branching have also showed that the interaction of GA and BR with auxin controls PR growth (Fu and Harberd, 2003) and promotes LR formation (Bao et al., 2004) respectively.

1.2.2 Nodulation

Nodules are formed through two closely coordinated processes: (i) the organogenic process, in which the nodule tissue is formed and (ii) the infection process, including the colonization of the bacteria inside the host plant (Madsen et al., 2010). The signal exchange between the two symbionts triggers nodule development. Flavonoids are released by the plant root to the rhizosphere as signal molecules to trigger the transcription of the nod genes in the rhizobia. Enzymes encoded by the Nod genes synthesise a second signal called the Nod factor. The rhizobia is recognised by plasma membrane-localised Lysine Motif-Receptor-Like Kinases (LysMs) in the epidermal cells. LysMs including *NFP* and *LYK3* in *M. truncatula* (Amor et al., 2003) and *NFR1*/*NFR5* in *L. japonicus* (Madsen et al., 2003) recognise rhizobial-derived Nod factors (Figure 1-4). Nod factors are small molecule

lipochitooligosaccharides (LCOs) (Oldroyd and Long, 2003, Limpens et al., 2003). Structural variation in the rhizobia-derived Nod factor and variation in the plant-derived signal flavonoids enable species-specificity in nodulation. Perception of the Nod factors by the LysM receptors initiates the symbiosis signaling pathway that activates transcriptional responses controlling nodule organogenesis, rhizobial infection and formation of symbiosomes inside the nodules (Kouchi et al., 2010).

Rhizobia enter the root through root hair cells. One of the earliest plant signaling events initiating symbiosis downstream of the symbiotic receptors is calcium oscillation (Ca^{2+} influx, immediately followed by Cl^- and K^+ effluxes). Calcium oscillation occur initially in the epidermal cells and later in the cortical cells (Oldroyd, 2013). These ion fluxes at the tip of root hair cells, trigger the deformation and curling of the root hair cell, forming structures known as shepherd's crooks. The rhizobacterial colony is then entrapped inside this structure (Mortier et al., 2012b). In *Medicago*, doesn't make infections2 (MtDMI2), which is a leucine-rich repeat receptor-like kinase (LRR-RLK), and MtDMI1, which is a nuclear potassium channel, are both involved in initiating calcium spiking to activate nodulation. It has been hypothesised that the frequency of epidermal calcium spiking codes for microbe specificity (Oldroyd and Downie, 2008), since in response to rhizobia these calcium spikes are highly regular (Sieberer et al., 2009), whereas in response to arbuscular mycorrhizae they can be highly irregular (Chabaud et al., 2011). However, other studies show that the calcium spiking between different types of microbial interactions are indistinguishable and that differences in calcium spiking are a reflection of the stage of the establishment of the symbiosis (Sieberer et al., 2012). The calcium spiking results in the activation of Calcium/calmodulin-dependent protein kinase (CCaMK), or DMI3 in *Medicago,* and its interacting protein IPD3, triggering the expression of the TFs NSP1, NSP2, ERN1 and NIN. These TFs activate the early nodulation (ENOD) genes that initiate the infection (Kosuta et al., 2008, Mortier et al., 2012b, Oldroyd, 2013) (Figure 1-4).

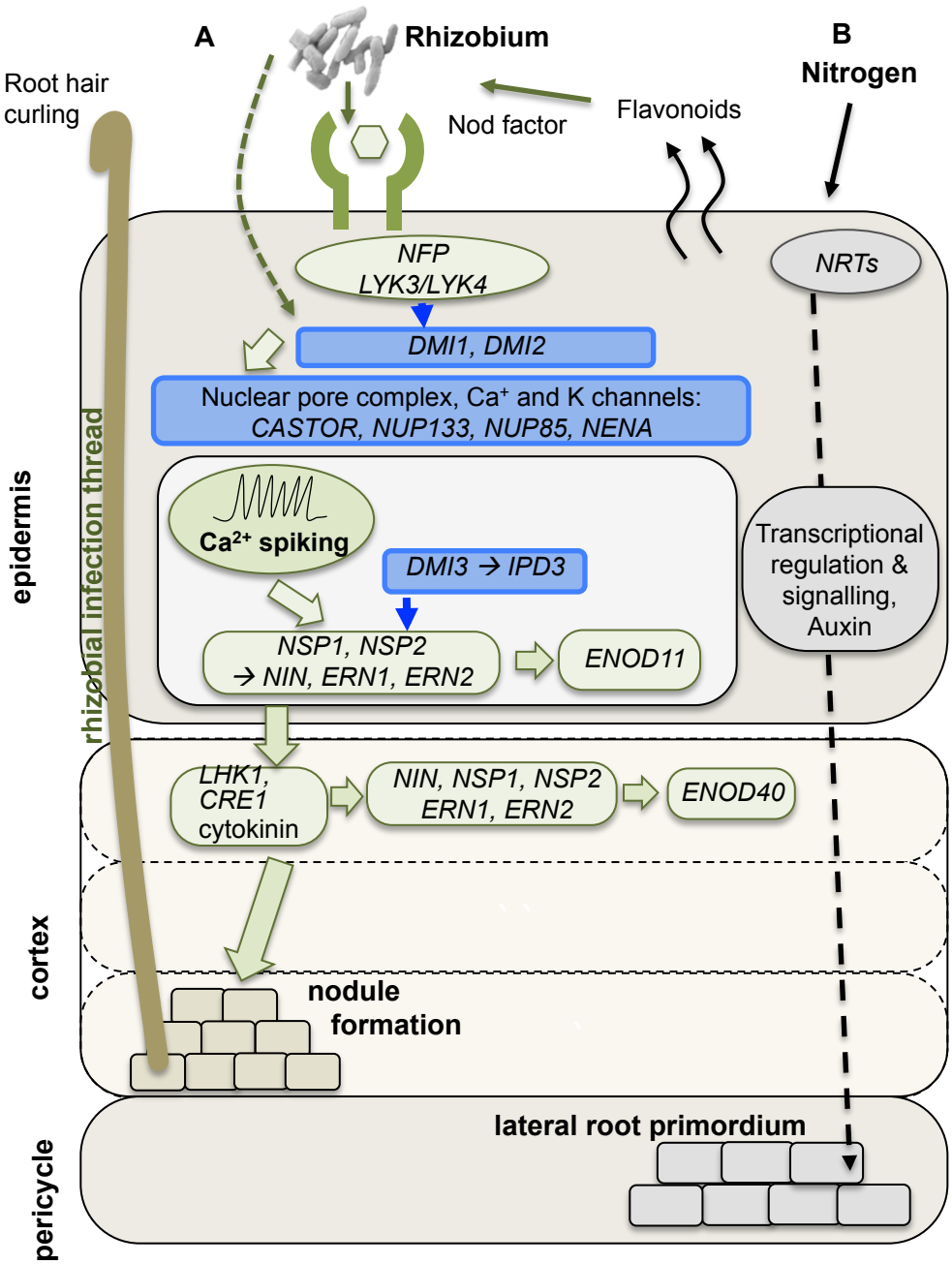


Figure 1-4: Overview of (A) nodulation, and (B) LR development in response to N. Rhizobia-derived small molecules are perceived by LysM receptors on the epidermal plasma membrane. Expression of NF signalling pathway leads to activation of calcium spiking in the nucleus and gene regulatory signalling between cell types that trigger nodule establishment in the inner cortex. LR primordia develop in the pericycle, regulated by signalling downstream of NRTs, transcriptional regulation and auxin signalling.

The encapsulated rhizobia divide multiple times and form a microcolony. It then enters the root and developing nodule through invasive structures, the infection threads that initiate at the site of the root hair curl and crosses the cortex to enter the developing nodule primordium. Prior to this, cortical cells dedifferentiate and divide to form the nodule primordia. Cytokinin signaling is one of the key components of nodule primordia formation (Crespi and Frugier, 2008, Frugier et al., 2008) and occurs downstream the NF signaling. In the epidermis, and downstream of DMI3, a mobile signal is generated and translocated to the cortex. This signal is perceived by cytokinin receptor CRE1. TFs ERN1, NSP2 and NIN are activated by signaling through response regulators inducing the expression of ENODs that induce cell division in the cortex (Gonzalez-Rizzo et al., 2006, Lohar et al., 2006, Oldroyd et al., 2011, Plet et al., 2011, Mortier et al., 2012b) (Figure 1-4). In indeterminate nodules such as in *Medicago* that have a tip-growing meristem, the nodules originate from inner cortex (nodule meristem). In the inner cortex and in the site of nodule initiation, rhizobia induce a local accumulation of auxin by inhibition of polar auxin transport below the infection site. Local inhibition of polar auxin transport downstream of *MtCRE1* contributes to the development of nodule primordia. *MtCRE1* is necessary for the inhibition of the polar auxin transport, but it is still unknown if *NSP2*, *ERN1* and *NIN* are also required for the inhibition of polar auxin transport (Plet et al., 2011, Mortier et al., 2012b).

The rhizobia are released inside the nodule primordia and through an endocytosis-like process, enter the cells. There they are surrounded by a plant-derived membrane and form the symbiosomes (nodule infection zone). Inside the symbiosomes they differentiate into bacteroids, which use a nitrogenase complex to fix atmospheric N_2 into ammonia (nodule fixation zone). The ammonia is then provided to the plant in exchange for carbohydrates (Jones et al., 2007, Mortier et al., 2012b).

1.2.3 Endogenous and external pathways controlling the extent of nodulation in legumes

Several regulatory controls exist to balance photosynthate and N exchange, in order to optimise whole plant growth and the ability of the root system to produce LRs for the acquisition of other nutrients. Nodule development is a costly process for the plant and C requirements are higher for N_2 fixation than for N assimilation . Thus, the overall tendency is towards using other sources of N such as nitrate and ammonium. At high concentrations of nitrate, total N uptake increases and nitrogenase activity decreases, which results in decrease of nodulation and N_2 fixation . Thus, plants are able to redirect C to root growth. N limitation stimulates nodule initiation and growth, which is followed by formation of new nodules if N starvation continues. Nodulation is regulated by different mechanisms according to the environmental condition of the plant.

Ethylene and ABA negatively regulate the number of nodules by affecting the calcium oscillation (Mortier et al., 2012b). The local ethylene-signaling pathway is involved in the regulation of Nod factor signaling and controls the number of nodules by impacting calcium spiking and restricting the infection (Gresshoff et al., 2009, Lohar et al., 2009). This inhibits root hair curling, reduces the calcium oscillation period, inhibits the bacterial infection and thus represses the expression of ENODs (Oldroyd et al., 2001). Consequentially, mutants impaired in ethylene sensitivity genes, such as the *M. truncatula* ethylene-insensitive hypernodulating mutant *sickle (skl),* which has a defect in the orthologous gene of *A. thaliana* ethylene-insensitive2 (*EIN2*) (Penmetsa and Cook, 1997, Varma Penmetsa et al., 2008), show an increased number of nodules. ABA is a negative regulator of nodule number in *M. truncatula* and *L. japonicus* (Ding et al., 2008, Biswas et al., 2009). At the early stages of nodulation, ABA reduces the nodule number by affecting calcium spiking in the NF signaling pathway, and at the later stage of nodulation, it suppresses cytokinin dependent nodule organogenesis (Ding et al., 2008).

Aside from local mechanisms controlling the number of nodules, the major pathway governing nodule number is Autoregulation of Nodulation (AON). Split root experiments show that this is a long distance signaling mechanism involved in the systemic inhibition of nodulation that is generated after root hair curling and prior to N fixation and initiation of visible cell division in cortex and pericycle (Suzuki et al., 2008, Li et al., 2009). After the activation of the cytokinin signaling, AON signaling is activated in the cortex affecting nodule primordia development. The number, activity and developmental stage of the nodules are factors governing the strength of AON (Mortier et al., 2012b). Studies on AON in pea have shown that AON could be activated in several nodule developmental stages (Li et al., 2009).

Nodulation related CLAVATA3/embryo-surrounding region (CLE) peptides, produced during nodule primordium formation and the nodule meristem development, are involved in the activation of AON, regulating the balance between cell division and differentiation (Mortier et al., 2010). In the AON pathway, along with the first nodule initiation and cell divisions in the roots, a long distance signal between root and shoot is initiated that is known as the Q signal. The Q signal is perceived in the shoot by a Leu-rich repeat receptor-like kinase (LRR-RLK). The shoot LRR-RLK is required for AON and is responsible for the systemic regulation of nodule number and density. It is similar to *Arabidopsis* CLAVATA1 (CLV1) and is encoded by *MtSUNN* (*Super numeric nodules*) in *M. truncatula* (Schnabel et al., 2005), *GmNARK* (*Nodule Autoregulation Receptor Kinase*) in *Glycine max* (Searle et al., 2003), *LjHAR1* (*hypernodulation aberrant root formation*) in *L. japonicus* (Krusell et al., 2002, Nishimura et al., 2002) and *PsSYM29* in pea (Krusell et al., 2002), suggesting a conserved AON mechanism across legumes. The nature of the Q signal is unknown but it is thought to be a CLE peptide (Reid et al., 2011a), and may be transported through the xylem (Okamoto et al., 2009, Mortier et al., 2010, Lim et al., 2011, Mortier et al., 2012a). Phylogenetic similarity between CLV1-like RLK receptors and putative peptide receptors such as CLV1, which are able to perceive CLE peptides, suggests that the Q signal may be a CLE peptide (Mortier et al., 2012b) and references within). Studies on *Glycine max*

have identified three different CLE peptides that are induced in response to rhizobial NF (*RC1* and *RC2*) or nitrate (NCI) treatment in a NARK (Nodule Autoregulation Receptor Kinase) dependent manner (Reid et al., 2011a). *GmRC1* is induced during initial signaling and cell division and has an expression pattern similar to *MtCLE13* and *LjCLE-RS1/2* (Okamoto et al., 2009, Mortier et al., 2010, Reid et al., 2011a). The expression of *GmRC2,* which is similar to *MtCLE12,* is upregulated at a later stage of nodule organogenesis and is associated with the emergence of more mature nodules, probably during the initiation of nodule meristem or in response to N fixation (Mortier et al., 2010, Reid et al., 2011a). In *GmNARK* mutants, over expression of *RIC1* and *RIC2* results in the complete inhibition of nodulation. Thus these CLE peptides may be acting as the Q signal in the AON pathway through NARK (Reid et al., 2011a).

The *CLV1-like RLK* expressed in the shoot, receives the Q signal and produces the Shoot Derived Inhibitor (SDI) signal. The identity of SDI is still unknown, but studies have shown that it is a small compound (<1 kDa), that is not a protein or RNA (Lin et al., 2010). This signal is transferred through the phloem to the root and inhibits cell division and the formation of any new nodules (Figure 1-5).

Crosstalk between AON and nitrate-regulation of nodulation is evident from the role of NARK and its orthologs in this regulation and the importance of CLEs in each. This is more directly evident in examples such as the nitrate tolerant symbiotic mutants of *Glycine max* (*nts*) and AON mutants that also exhibit a supernodulation phenotype at high nitrate levels. In *L. japonicus, LjCLE-RS2* responds to both nitrate treatment and rhizobia inoculation and systemically induces regulation of nodulation when overexpressed (Okamoto et al., 2009). In *Glycine max*, the N-Induced CLE peptide, *NIC1,* is induced locally in the root in response to nitrate and is involved in the local inhibition of nodulation in a NARK dependent manner (Reid et al., 2011a). Local and systemic regulation of nodulation is affected in *GmNARK* mutants

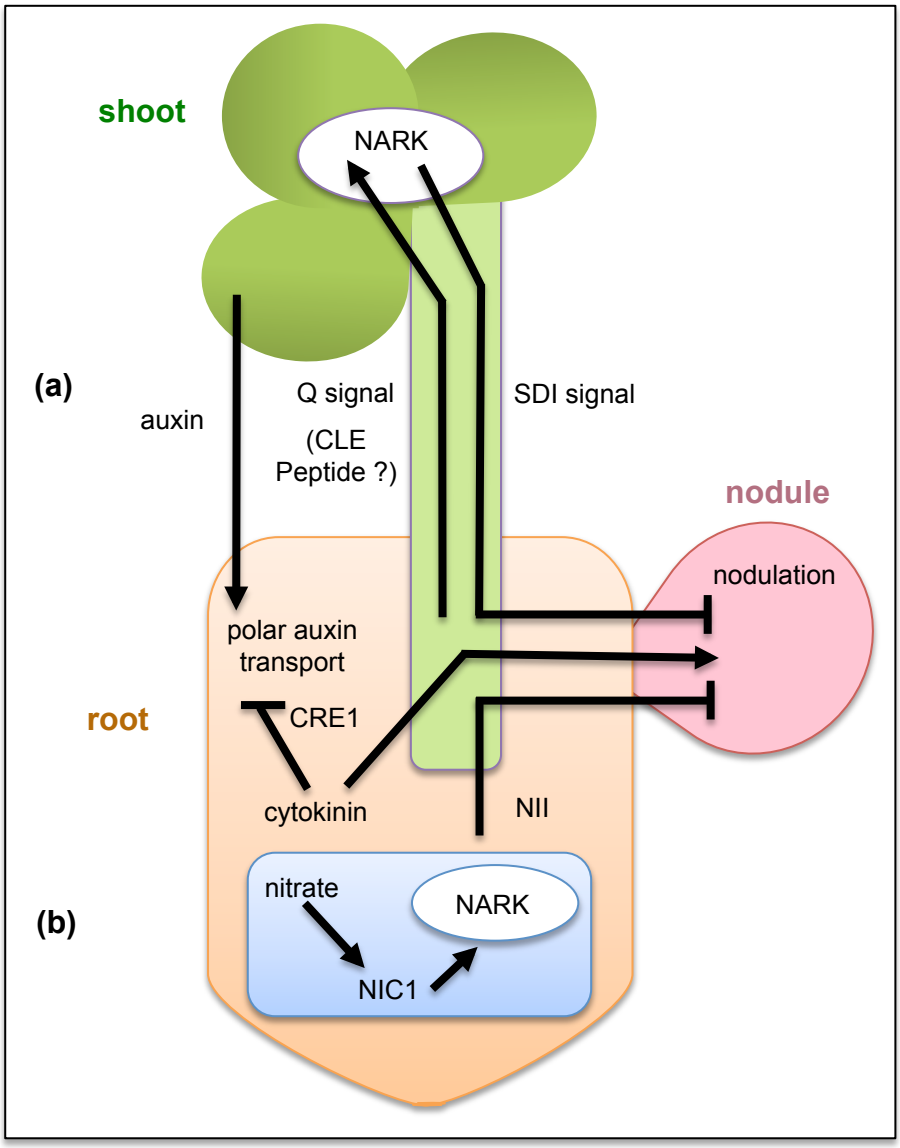


Figure 1-5: A model for control of nodule number in legumes. The number of nodules is regulated through both local and systemic regulatory pathways. (a) Auto regulation of nodulation (AON) pathway is a systemic pathway, which is activated at early steps of nodulation. The exact identity of the components of this signaling pathway is still unknown but evidence so far suggests that a long distance signal (Q), most likely a CLV3/EMBRYO SURROUNDING REGION (CLE) peptide is transferred from root to shoot and is perceived by the shoot LRR-RLK, NARK in pea. A Shoot Derived Inhibitor (SDI) signal is then transferred from shoot to root to inhibit nodulation. (b) In parallel, a local signaling pathway inhibits nodulation under high N concentrations. This signal may be a N-induced CLE1 (NIC1), which is perceived by the root NARK that in turn sends a second signal, the SDI-like nitrate induced inhibitor (NII) to inhibit further nodulation. Cytokinin signaling interacts with AON to control the number of nodules.

(Reid et al., 2011b). This indicates that in *G. max* this gene may be common to both systemic (AON) and local (nitrate induced) regulation of nodulation. Grafting experiments have shown that *NIC1* is perceived by root-localised NARK during the local regulation of nodulation in roots by nitrate (Reid et al., 2011a). A SDI-like nitrate induced inhibitor (NII) is then produced, which inhibits the formation of nodules (Figure 1-5). In *L. japonicus LjHAR1* (*hypernodulation aberrant root formation*) mutants, lacking the ability to recognize the LjCLE-RS2 peptides in the presence of nitrate, could explain the reason for nitrate tolerance in these plants (Okamoto et al., 2009).

Cytokinin signaling is well established in regulating nodule organogenesis (Plet et al., 2011, Heckmann et al., 2011). More recently several studies have shown that there is a correlation between cytokinin signaling and AON (Mortier et al., 2010, Mortier et al., 2012a, Saur et al., 2011, Takahara et al., 2013). More directly, *Too much love* (*TML*) may act downstream of the cytokinin receptor *LHK1/CRE1* and thus the F-box protein TML may be one of the first components in AON to regulate the number of nodules (Takahara et al., 2013).

1.3 Impact of reducing N requirements for crops

Our society faces a major challenge in meeting the demands of an expanding and developing global population. According to the latest world population report from the United Nations (2012), the global population is estimated to reach 9.6 billion by 2050. In combination with the changes in environmental conditions such as water availability created by the climate change due to emission of greenhouse gases that cause a temperature rise, there are massive implications for the ability to deliver food security. Higher temperatures could have serious effects on agriculture by reducing crop yield. Analysis of detailed modeling of crop growth under climate change by simulating future climate conditions suggests that agriculture and human well-being will be negatively affected by climate change (Nelson, 2009).

In the UK, The Global Food Security Program (http://www.foodsecurity.ac.uk/programme/about-the-programme.html) is to provide the growing global population with sustainable food supplies using less land and resources. In accordance with the global security food program it is important to discover new ways and technologies to provide sustainable food supply. Having a better understanding of the ways that plants respond to stress could help us manage and improve crop productivity.

As one of the main mineral nutrients required by plants to complete their life cycles, the availability of N is central to global food security. The unreactive N_2 is abundant and makes up to 80% of the earth atmosphere (Sanhueza, 1982) but the reactive oxygen compounds (including oxidized and reduced N compounds, such as nitric acid, ammonia, nitrates, ammonium and organic N compounds) that are usable by plants and animals are generally scarce in the natural environment. This is a key constraint making it impossible to provide food for the increasing world population only relying on the N natural cycle. That is why this population is highly dependent on the application of nitrogenous fertilizers, manufactured through the Haber-Bosch process (Marschner, 1996). Although use of fertilizers enables increased plant productivity, agricultural use of nitrogenous fertilizers has significant environmental and economic consequences and we cannot afford to use them at the level we are currently using. Nitrate in fertilizers are washed off into watercourses or leached through soil into groundwater polluting the waters. This affects natural habitats and human health. Emissions of ammonia and nitrous oxide to the atmosphere have serious impacts on biodiversity and climate change. Application of nitrate fertilizers is currently a major driver in agricultural energy consumption and production costs. Production of inorganic fertilizers such as N fertilizer accounts for 1.2% of the world energy usage (Swaminathan and Sukalac, 2004). Prices for these fertilizers will continue to grow as energy prices increase. Excessive usage of nitrate fertilizers by the farmers is a major source of carbon dioxide emission from agriculture (about 50%) and contributes to emission of greenhouse gases.

Understanding how plants manage and balance interactions with different microorganisms is more important than ever in this new era of attempting to transfer the ability to nodulate into non-legume species such as rice and wheat. This could be one of the ways for breaking our dependency on costly N fertilizers. If we can better understand the regulatory genes that control nodulation, and identify orthologous genes that could potentially control symbiosis in non-legumes we could inform new strategies for plant development. However, to ensure that this approach is successful we need to understand how nodulation affects development of other root organs such as LRs that are needed to take other limiting nutrients such as phosphorus. This is key since plants need to balance the resources required to form organs against the payback from nutrient uptake.

1.4 Aim and Objectives

This study tried to investigate the balance between LR formation and nodule development, and the interaction with N availability in *M. truncatula*. This was done by studying phenotypic and gene expression changes in root under low and high N concentration and in response to rhizobial inoculation. The ultimate goal of this study was to investigate these gene expression changes at root tissue level, in pericycle and cortex. This study aimed to:

Chapter 3: Phenotypic crosstalk between nodulation and LR development

Using *Medicago* as a suitable legume model plant because of its ability to form nodules and its available genetic resources, phenotypic analysis of RSA was carried out. The number of nodules and key root parameters were measured to quantify the effect of N availability, presence of rhizobia and the ability to nodulate. Four *Medicago truncatula* ecotypes were analysed to identify consistent regulatory effects in *Medicago*.

Chapter 4: Expression analysis to identify genetic control of developmental crosstalk

Whole genome expression profiling using microarrays was carried out to answer key questions about the plant behavior through N and rhizobia at early hours after treatment with high NH_4NO_3. Early responses to NH_4NO_3 were studies 2 and 6 h post treatment with high NH_4NO_3 (5 mM) in rhizobia inoculated and mock inoculated A17 (here known as the wild type) plants grown at low NH_4NO_3 (0.1 mM) concentration. Gene expression changes of whole root samples were studied to identify genes involved in early responses (2 and 6 h) to NH_4NO_3 treatment in the presence and absence of rhizobia. Since nodules are initiated in the cortex and LRs in pericycle, transgenic plants expressing GFP in pericycle or cortex were generated to study gene expression changes at cell-specific level.

Phenotypic studies on root architecture changes under low and high NH_4NO_3 concentrations showed that at high NH_4NO_3 presence of rhizobia affected root growth. Thus the effect of rhizobia under high or low NH_4NO_3 treatment on rhizobia inoculated samples grown at high NH_4NO_3 was studied to identify significantly affected genes.

Chapter 5: Analysis of hypernodulation mutant *sunn-1* implicates balancing genes to regulate LR development

M. truncatula A17 and its hypernodulating mutant *sunn-1* that is impaired in the long distance AON-related shoot signaling controlling the number of nodules, show opposite root phenotypes in responses to N and rhizobia. Thus they were used as model plants to see how A17 and *sunn-1* were affected by the presence of rhizobia and how they responded to NH_4NO_3. For this genome expression changes 6 h post treatment with low or high NH_4NO_3 was studied in whole root samples of rhizobia inoculated or mock-inoculated plants grown at low NH_4NO_3. This experiment enabled to identify some of the genes affected by rhizobia at low or high NH_4NO_3 and also the effect of low and high NH_4NO_3 in the presence or absence of rhizobia on gene expression in A17 and *sunn-1* backgrounds. It also identified the genes that were affected by *SUNN* in the presence or absence of rhizobia under low or high NH_4NO_3

treatment. The genes showing significant expression changes under these conditions could be also some of the genes involved in AON and the balance between LR and nodule development. To have a better understanding of how these genes are controlled promoters of genes for transcriptional motifs were studied.

Impact of this PhD thesis

The phenotypic and transcriptomic data generated from this study was integrated together to develop a systems-level understanding of N use efficiency in *Medicago*. The data gathered from this project could be used to develop new tools, technologies and materials to improve N resource-use efficiency in plants and address one critical aspect of food security.

Chapter 2

Materials and Methods

2.1 Plant material

Seeds were of *Medicago truncatula* cv. Jemalong line A17 (sequenced reference accession, denoted here as 'wild type') and available *Medicago truncatula* accessions from the IGER seed bank (Aberystwyth, http://www.igergru.ibers.aber.ac.uk) *M. truncatula* cv. Jemalong 2HA, *M. truncatula* ssp. *tribuloides*, *M. truncatula* Gaertner, *M. truncatula* var. longispina. Also the A17 background hypernodulating mutant *sunn-1 (super numeric nodules)* (Schnabel et al., 2005) that was kindly provided by Giles Oldroyd (John Innes Centre).

2.2 Plant growth

2.2.1 Germination of *Medicago* seeds

2.2.1.1 Seed extraction from pods

Seedpods were placed on a corrugated rubber mat and a plasterer's hawk with handle was used to crush them with a circular movement, releasing seeds between the corrugations and protecting them from damage.

2.2.1.2 Scarification

Seeds were scarified either mechanically or chemically depending on the quantity of the seeds.

Mechanical scarification was used for small quantities of seed (n<50). In this method seeds were rubbed gently between sheets of fine graded sand paper until there were visible signs of scratches, indicating scarification.

Chemical scarification was used for larger quantities of seed (n>50). Seeds were incubated in concentrated H_2SO_4 in a 50 mL plastic centrifuge bottle on a shaker (150 rpm) for about 8 min. Appearance of black colouration on the seeds was a sign of scarification. Excess H_2SO_4 was removed using a pipette and seeds were rinsed (3-4 times) with sterile water.

2.2.1.3 Surface sterilization

Seeds were incubated in 50:50 (v:v) bleach:water (3% sodium hypochlorite) and agitated intermittently for 3 min, then rinsed in sterile water for 4 times each time 2 min.

2.2.1.4 Seed germination

Sterile seeds were sown on 1.5% agar in sterile 9 cm single vent petri dishes (Greiner Bio-one) sealed using 1.25 cm x 9.1 m microporous tape (3M™ Micropore™) and imbibed for 1 day in dark at 4°C for optimal synchronization of germination. In the cases were seed dormancy had to be broken, similar cold treatment was applied to the seeds for 3 days. Seeds were then incubated inverted (to promote straight radicles) in a growth chamber of 25°C under fluorescent lights (16/8 hr light/dark photoperiod) for 5 days.

2.2.2 Growth on pouch paper overlays on agar growth medium plates

Changes in *Medicago* root architecture in response to external nitrate concentrations and rhizobial inoculation, were determined using growth pouches (CYG™ Germination Pouch, West St. Paul, MN, United States) overlaid on agar containing modified Fahräeus medium (Vincent, 1970). The modified N-free Fahräeus medium contained the following macronutrients (0.5 mM $MgSO_4.7H_2O$, 0.7 mM KH_2PO_4, 20 mM Ferric citrate, 0.4 mM

$Na_2HPO_4.2H_2O$ and 0.9 mM $CaCl_2$) and micronutrients ($MnSO_4$, $CuSO_4$, $ZnCl_2$, H_3BO_3 and Na_2MoO_4 each 1 mg mL^{-1}). N was added as NH_4NO_3 at varying rates depending on the experiment (0, 0.1, 0.2, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 5, 10, 15 or 20 mM). The pH was adjusted to 6.5 using KOH and 1.5% (w:v) agar was added before sterilization by autoclaving. After autoclaving, 0.075 µM (S)-trans-2-Amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride (AVG) was added to the cooled (around 60°C) medium. The medium was then poured into sterile square Petri dishes (120 mm × 120 mm x 17 mm) with vents (Greiner Bio-one). AVG is an ethylene inhibitor (Peters and Crist-Estes, 1989) and it is needed for enhancing nodulation in the hypernodulating mutant *sunn-1.* To make the experiments comparable AVG was added to the growth medium in all experiments.

Growth pouches were cut in the shape of the plates and autoclaved before soaking them in liquid modified Fahräeus medium (same as above but without agar) and placed on to the modified Fahräeus agar plates (Figure 2-1). In sterile conditions, 6-7 germinated seedlings were transplanted between two layers of growth pouch in each plate and the plates were sealed using 1.25 cm x 9.1 m microporous tape (3M™ Micropore™). Plates were placed into black plastic bags so that only the roots were covered (for decreasing light exposure and mimicking soil conditions) and grown vertically in the growth chamber (25°C, 16/8 hr light/dark) for 4 days. Seedlings were then inoculated with *Sinorhizobium meliloti* (as in 2.3.1.3) or mock inoculated using liquid modified Fahräeus medium, and then incubated in the growth chamber for 14 days.

2.3 Treatments

2.3.1 Rhizobial inoculation and nodulation

Sinorhizobium meliloti kindly provided by Giles Oldroyd (John Innes Centre) was used as the rhizobium symbionts strain for *M. truncatula* nodulation.

Figure 2-1: The growth pouch system for growing *Medicago*. Seedlings would be placed between two layers of growth pouch (top layer not shown in the image) on modified Fahräeus medium. The image shows 14-day mock-inoculated seedlings of A17 grown at low NH_4NO_3 .

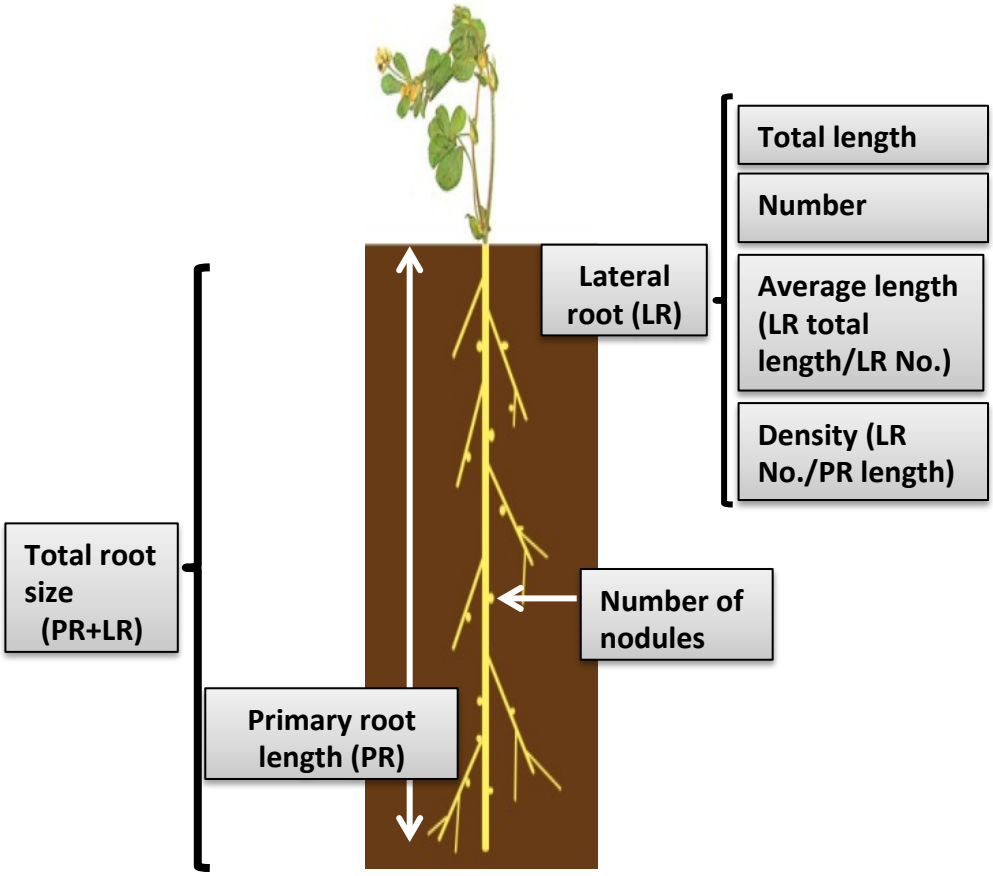


Figure 2-2: Different root attributes measured for quantifying root architecture response to NH_4NO_3 .

2.3.1.1 Growing the rhizobia

S. meliloti was cultured on agar TY/Ca_2^+ medium (Journet et al., 2006). This medium was prepared by adding 5 g l^{-1} Bacto-tryptone, 3 g l^{-1} Yeast extract and 1.2% (w:v) bacterial agar into sterile deionized water. The medium was then autoclaved and 6mM $CaCl_2$ was added to the cooled (around 60°C) medium. Rhizobia was streaked out onto the solid medium and incubated for 3-4 days at 28°C.

2.3.1.2 Preparing rhizobial solution for inoculation

Rhizobial solution for inoculation was prepared by inoculating 50 ml of liquid TY/Ca_2^+ medium with a subculture of *S. meliloti* or *Agrobacteria tumefaciens* (for plant transformation) and incubated for 26 hr at 28°C while shaking at 220 rpm to an OD600 of 1-2. Cells were then harvested by centrifugation (4000 rpm, 10 min, 4°C) and re-suspended in 40 ml liquid modified Fahräeus medium (supplemented with the same concentration of NH_4NO_3 as the modified Fahräeus agar medium) or liquid TY/Ca_2^+ medium in the case of plant transformation to a final OD600 of 0.8 (Zhou et al., 2004).

2.3.1.3 Inoculating roots with *S. meliloti*

At dawn in the light/dark plant growth chamber cycle, plants were removed from the growth chamber and 2 mL of rhizobial solution was added evenly to the roots of the seedlings under sterile conditions. Mock inoculation was also carried out in the same way, using 2 mL of sterile medium. Roots were then covered again with the growth pouch layer and the plates sealed with micropore tape and placed back into the growth chamber for 14 days.

2.3.2 Ammonium nitrate treatment

Fourteen days post inoculation (dpi) roots were treated with 2 mL per plate of liquid modified Fahräeus medium supplemented with NH_4NO_3 (low or high concentration depending on the experiments as explained in the following chapters) and with rhizobia inoculation or without (mock) inoculation.

For subsequent microarray experiments, growth pouches with seedlings were infiltrated with the solutions for 5 seconds. In all cases, growth pouches with seedlings were placed on new plates of Fahräeus agar medium supplemented with the same NH_4NO_3 concentration as the treatment. Treated plates were kept in the incubator for 2-6 hours (for the microarray experiments) or up to 16 days (for analysis of root architecture at the cellular level).

2.4 Phenotyping root architecture and nodule development

2.4.1 Quantifying root system architecture

Plant roots were scanned using a flatbed scanner at highest resolution at 14 dpi and root attributes were measured (Figure 2-2) The number of LRs and nodules (for rhizobia inoculated seedlings) were counted and PR length and LR length was measured using ImageJ software (Schneider et al., 2012). Average of total LR length and average of LR length (sum of LR length /number of LRs per plant) was calculated from the ImageJ measurements. Total root size was expressed as the sum of PR and total LR length, and LR density as the number of LRs per cm of PR. An individual biological replicate consisted of measuring 6 seedlings on each plate and 2 plates.

2.4.2 Data analysis

Logarithmic transformation or square root transformation was used to normalize the data. The effect of different NH_4NO_3 concentrations and rhizobial inoculation between rhizobia inoculated and mock-inoculated samples were compared applying the restricted maximum likelihood (REML) statistical model using the Genstat software (VSNInternational, 2011).

2.4.3 Measuring N in root and shoot

Seedlings of A17 and *sunn-1* were grown on Fahräeus medium supplemented with low (0.1 mM) or high (5 mM) NH_4NO_3 and inoculated with rhizobia or mock-inoculated with liquid modified Fahräeus medium

supplemented with the same concentration of NH_4NO_3 as their growth condition. At 14 dpi, the seedlings were rinsed with sterile water to remove adhering nutrient solution and blot dried to remove excess moisture before measuring the fresh weights. The wet and dry weight of roots and shoots of 3 biological replicates (consisting of 6 seedlings on each plate and 3 plates for each experiment, n=54) was measured using a four decimal place balance (Sartorius CP225D Dual Range Analytical Balance). They were then transferred to plastic tubes and oven dried (80°C) for 3 days before dry weights of the samples were measured.

Dry samples in three biological replicates were pooled together, milled to 2 mm grading and prepared as follows for free NO_3, total N and total P measurement. The samples were pooled together because sufficient dry samples were not available to carry out the mineral measurement assays thus the resulting data were not replicated.

2.4.3.1 Measuring free nitrate in root and shoot

Dried and ground root or shoot tissue was measured (0.1 g) on a four decimal place balance into 50 ml centrifuge tubes. Reverse osmotic water (25 ml) was added to the samples and placed on a bottle shaker to shake (200 rpm) for 30 min. They were then filtered through no. 1 filter paper into scintillation vials. Free nitrate was then measured using a flow injection analyser (Foss FiaStar System). The measurement values were multiplied by sample weight and divided by volume of extractant (25 mL) resulting in NO_3 concentration expressed as 'N'. This was multiplied by NO_3 molecular weight and divided by N molecular weight to determine the amount of NO_3 in each sample.

2.4.3.2 Measuring total N and P in shoot

Sulphuric acid digest by the Kjeldahl method was used to measure total N and P in shoot. About 0.1 g of oven dried and ground shoot was weighed on a four decimal place balance into a digestion tube. Anti-bumping granules

were added for smooth boiling. In the fume cupboard 2 mL digestion acid (sulphuric acid) was added and the tubes were covered with a polythene bag and left overnight. Then, 1 mL of 30% hydrogen peroxide was added and tubes were loaded into a heating rack and heated using the Gerhardt digestion system. Tubes were left to cool down between the programs prior to adding a further 1 mL of hydrogen peroxide. The tubes were then allowed to cool and 48.6 ml reverse osmotic water was added and samples mixed well on a vortex. The samples were transferred to scintillation vials and analysed on the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP).

2.4.4 Analysing root architecture changes at the cellular level

A17 seedlings were grown on plates containing modified Fahräeus medium supplemented with low NH_4NO_3 (0.1 mM). The seedlings were rhizobial inoculated or mock inoculated. At 14 dpi they were treated with high or low NH_4NO_3 (Figure 3-7). At 0, 4, 8, 12, 16-day time points, roots of the seedlings in three replicates were stained (section 2.4.4.1) and viewed at 10X under differential interference contrast (DIC) using an Olympus BX51 microscope.

Nodule primordia and nodules were counted (Lohar et al., 2006) (Figure 3-8) and the developmental timescale of LRs was measured using a comparison to defined LR development stages in *M. truncatula* (Herrbach et al., 2013) according to the following categories (Figure 3-6): (i) stage Ia to III LRP (Figure 3-6 A-D), (ii) stage IV to V LRP (Figure 3-6 E-F); (iii) emerging LRP (Figure 3-6 G) and (iv) fully emerged LR (Figure 3-6 H). Roots in class (i) and (ii) were scored as stage i and ii "initiating" LRP and roots in classes (iii) and (iv) were scored as "emerging" LRP and "emerged" LR respectively. LR length was measured using ImageJ.

2.4.4.1 Root tissue staining

Root tissue was incubated in 1% Periodic acid (10 g/L Periodic acid) for 40 min at room temperature. They were then rinsed with water and incubated

in Schiff reagent containing propidium iodide (100 mM Sodium metabisulphite, 0.15 M HCl, 100 µg mL^{-1} propidium iodide added fresh just before use) for 1-2 hours at room temperature until tissue was visibly stained (pink). Samples were transferred onto microscope slides, covered with chloral hydrate solution (4 parts (w/v) chloral hydrate, 1 part (v/v) glycerol, 2 parts (v/v) deionised H_2O) and incubated in a closed environment (to prevent drying out) at room temperature. Excess chloral hydrate solution was removed and samples were mounted in several drops of Hoyer's medium (30 parts (w/v) gum arabic, 200 parts (w/v) chloral hydrate, 16 parts (v/v) glycerol, 50 parts (v/v) deionised H_2O) and covered with a cover slip. Slides were left undisturbed for 3 days allowing mounting solution to set (Truernit et al., 2008).

2.5 Nucleic acid techniques

2.5.1 DNA and RNA extraction

2.5.1.1 Total RNA extraction from whole root tissue

Whole roots from three independent biological replicates were cut using a sterile surgical blade and frozen in liquid N in 2 mL sterile microcentrifuge tubes. Samples were stored at -80°C or RNA isolated immediately. Frozen roots were ground thoroughly in liquid N using an electric drill. The drill bit was first submerged in liquid N for 5 s and used for grinding the samples inside the liquid N-cooled microcentrifuge tubes. After root samples were completely crushed into powder, 450 µL Buffer RLT (from the Qiagen RNeasy Plant Mini Kit) was immediately added and then samples were ground for another 10 sec. They were then mixed by vortexing and kept on ice for the remainder of the procedure.

Total RNA was extracted using the Qiagen RNeasy Plant Mini Kit and eluted using RNase-free water all according to the manufacturer instructions.

RNA was quantified using a spectrophotometer (NanoDrop 1000, NanoDrop Products, Wilmington, DE, USA). The Nanodrop stage was cleaned with 70% ethanol before and between loading of each 1.2 µl sample.

The spectrophotometer was set to measure RNA quantities against a blanked measurement of 1.2 µl RNase-free water (from the Qiagen kit) and blanking was repeated after measuring every 10 samples.

Total RNA samples were then treated with the TURBO DNA-free kit (Life Technologes) following the manufacturer's instructions.

2.5.1.1.1 Verifying lack of DNA contamination

A polymerase chain reaction (PCR) was performed using tubulin as reference gene primer. The samples were then analysed using gel electrophoresis (detailed in 2.5.2) for lack of DNA contamination, compared to PCR products from a positive control reaction that used gDNA as template.

2.5.1.2 Extraction of plant genomic DNA

For fast extraction of DNA for PCR (Edwards et al., 1991), each leaf sample was freeze dried in liquid nitrogen and crushed into powder using an electric drill followed by addition of 400 µL Edwards extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, pH 8, 0.5% SDS). All steps were carried out at room temperature. Samples were vortexed and centrifuged at 5,600g for 1 min. 200-300 µL of supernatant was transferred to a microcentrifuge tube and an equal amount of 100% isopropanol added. This was mixed and incubated at room temperature for 2 min prior to centrifuge at 4,000g for 5 min. The supernatant was removed and the pellet air dried at room temperature, before beingresuspended in 50-100 µL sterile TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8).

2.5.2 PCR and gel electrophoresis

For polymerase chain reaction (PCR) a mastermix of 5 µL 2x MyTaq red mix (Bioline Reagents Ltd, London, UK), 0.3 µL dimethyl sulphoxide (DMSO), 0.3 µL of each primer (forward and reverse) and 3.7 µL RNase free water was made and mixed by vortexing. 9.6 µL of master mix was added to 0.4 µL of RNA sample in 200 µL tubes. Tubes were briefly vortexed and briefly

Table 2-1: Program for PCR

Step	Temperature	Duration	Number of Cycles
Initiation	94 ºC	2 min	1x
Denaturing	94 ºC	30 s	30-35x
Annealing	58 ºC	30 s	
Extension	72 ºC	30 s per 500 bp	
Completion	72 ºC	5 min	1x
Cooling	4 ºC	Up to 20 min	1x

centrifuged and placed in a thermocycler (Applied Biosystems, Invitrogen, Carlsbad, CA, USA). The thermocycler was set on a program as in Table 2-1.

The samples were then analysed using agarose gel electrophoresis. 1% w/v agarose was mixed with 100 ml 1xTAE buffer (Tris base, acetic acid and EDTA, prepared from a 50x stock solution). The agarose was melted in a microwave oven by heating for 60 s and cooled prior to adding 3 µl ethidium bromide (EtBr). This mixture was poured into a gel mould (Thistle Scientific, Glasgow, UK) with a toothed plastic comb. After the gel was set, the comb was removed and the gel was placed in a gel running tank (Thistle Scientific). 1xTAE buffer was added to cover the gel and the PCR reactions (10 µl) were pipetted into the wells alongside 2 µl of 1Kb DNA HyperLadder I (Bioline). The gel was run for about 90 min at 100-120 V. The gel was viewed under ultraviolet light using a G:Box gel imaging system (Syngene International Limited, Biocon Limited, Bangalore, India).

2.5.3 Synthesis of cDNA from RNA

cDNA synthesis and amplification was carried out using the Ovation Pico WTA System (NuGEN Technologies Inc., San Carlos, CA, USA) following the manufacturer's instructions. The cDNA was then purified using Qiagen Qiaquick PCR purification kit following the manufacturer's instructions. The cDNA was quantified using a Nanodrop set to measure DNA quantities and 260nm/280nm and 260nm/230nm measurements were recorded.

2.5.4 Microarray experimental methods

A Roche-Nimblegen (Roche Applied Science, Penzberg, Upper Bavaria, Germany) microarray platform was used to quantify transcript abundance in *Medicago* whole root samples. This platform used a 12x135k probe array with a custom design (OID36783) for the *M. truncatula* Mt3.5 genome; each of 47,530 genes was measured with 2-3 unique 60mer oligonucleotide probes (total of 14,6171 probes) per gene.

A 0.5 µg sample of purified and amplified cDNA was amplified and labeled with Cy3 dye for single channel microarray analysis using the Nimblegen one-color DNA labeling kit according to the manufacturer's instructions. After Nanodrop quantification, 4 µg of the labeled cDNA was hybridized using the Nimblegen hybridization kit onto the array according to manufacturer's instructions. Samples in 3 biological replicates and 3 technical replicates were randomized using a bioinformatic tool (http://www.randomizer.org/) prior to loading on the slides.

The slides were scanned on a Nimblegen MS 200 microarray scanner using the Nimblegen MS 200 Data Collection Software. To measure the fluorescence intensities of the microarray, Cy3-labeled samples were excited using green laser (532 nm wavelength). To gain an overall overview of the microarray quality, a quick scan of 20 µm resolution was performed, and if hybridization was confirmed then the slide was scanned at 2 µm resolution. Scan and data collection were carried out as per manufacturer's instructions. The data was collected as XYS files (raw probe level data by coordinates on the microarray), supplying the array coordinates (from the array design file) and observed intensities (x, y and signal).

2.6 Statistical analysis of microarray data

2.6.1 Normalization, quality control and summarization

Microarray analysis was conducted in the R software environment (R Core Team, 2012). An annotation package associated with the array design was built by using the pdInfoBuilder package (Falcon et al., 2012) and the .ndf design file provided by the manufacturer. The XYS files were identified and imported into the R session using the Oligo package (Carvalho and Irizarry, 2010). The quality of the data was checked by generating boxplots, smooth histograms and heatmaps of Pearson correlation coefficients between all arrays of the raw data; these were generated using the boxplot and hist methods from Oligo (Carvalho and Irizarry, 2010) package for R. For background adjustment, quantile normalization and summarization (to gene

level by median polish), Robust Multi-Array Averaging (RMA) was performed using the Oligo package for R (Carvalho and Irizarry, 2010).

2.6.2 Identifying differentially expressed genes

The quality of the arrays was rechecked (using boxplots, smooth histograms and heatmaps). To identify differentially expressed genes, a GaGa model with a significance cutoff of P=0.05 (Russel, 2011) or moderated t-statistic with a cutoff of P=0.001 in limma package for R (Smyth, 2004) was used. In the case of using the GaGa model, first different patterns comprising of all the possible comparisons between the treatments (in 3 or 2 technical replicates) were generated in R. These patterns were then used in the GaGa model to identify differentially expressed genes. Differentially expressed genes were clustered and fold changes of the log2 expression values were calculated for further analysis.

2.6.3 Identifying clusters of differentially expressed genes

Differential expressed genes were clustered using hierarchical clustering with an average linkage and Pearson correlation. Silhouette widths were plotted in MATLAB for each hierarchical tree and used to determine where to cut the trees and define clusters (MATLAB function for clustergram, MATLAB, The MathWorks http://www.mathworks.com). Heatmaps were generated with the heatmaps.2 function from gplots package (Warnes et al.) for R. Colors were set using the RColorBrewer (Neuwirth, 2007) package.

2.6.4 Assigning differentially expressed genes to functional categories

The MapMan software (version 3.5.1R2) (Thimm et al., 2004) was used to assign differentially expressed genes with FC > 2 or FC < -2 into functional categories defined by MapMan. This was done based on assessing similarities with *Arabidopsis* proteins (Thimm et al., 2004). Wilcoxon Rank Sum Test was used to predict functional categories (BINs) that show a different behaviour in terms of expression profile compared to all the other remaining BINs.

2.6.5 Motif analysis

MEME-LaB tool (Brown et al., 2013) was used to search for putative motif sequences within groups of co-expressed genes. The software compares the input datasets with 2 kb upstream promoter sequences of the genes. To generate this file genomic locations for all *Medicago* genes in the Mt 3.5v5 assembly was indexed against the entire *Medicago* genome sequence (Young et al., 2005, Cannon et al., 2006) and a FASTA file of 2 kb upstream promoter sequences for each gene was generated. This was then hard masked using the RepeatMasker software version 4.0 (Smit et al., 2010) that screens the sequences for interspersed repeats and low complexity sequences. The output is a modified version of the query sequence in which all the annotated repeats have been masked.

MEME-LaB analysis was run for each dataset with different promoter maximum lengths (200, 500 and 1000 bp). Other parameters were set as: promoter minimum length = 100 bp, minimum motif width = 6 and maximum motif width = 12. MEME was run independently on each group and for each group of co-expressed genes MEME-LaB generated a max of 5 putative motifs.

2.7 Generating *Medicago* transgenic lines expressing GFP

Transgenic plants expressing GFP in pericycle or cortex were generated via callus *Agrobacterium tumefaciens*-mediated plant transformation from *M. truncatula* A17 as explained in (Zhou et al., 2004). Mature seeds were sterilized and placed on 100cm diameter x 25cm deep sized round Petri dishes containing germination medium (3.2 g L^{-1} Schenk and Hildebrandt (SH) basal salt mixture (Sigma), 1.01 g L^{-1} SH vitamin powder (Sigma), 20 g L^{-1} Sucrose, 1 mg L^{-1} 6-Benzylaminopurine (BAP) in 0.8% agar (w/v), pH 5.8). Seeds (15 seeds per plate) were incubated for 4 days at 25 °C under fluorescent light (16/8 h light/dark); this incubating condition was used in all the transformation steps. After 4 days each seedling was transected 1-2 mm below the cotyledons to remove the radicle and most of the hypocotyl. The

remaining tissue was then bisected so that each explant had one cotyledon and half of the embryonic axis. Explants were inoculated with *Agrobacterium tumefaciens* carrying the pBGWFS7 vector in which each of four gene promoters (Medtr7g118460, Medtr4g018260, Medtr5g093170, Medtr8g121420) drove GFP expression. These genes were selected since they were found to be orthologous, based on a reciprocal best BLAST search, to cell type specific genes in *Arabidopsis thaliana* (Gifford, Unpublished results). Explants were immersed in the bacterial solution (prepared as described in section 2.3.1.2) and shaken gently for 30 min. They were blotted dry on filter paper to remove excess bacteria and placed adaxial side face-up on 100cm diameter x 25cm deep sized round Petri dishes containing co-cultivation medium (3.2 g L^{-1} SH salts, 1.01 g L^{-1} SH vitamins, 20 g L^{-1}sucrose, 400 mg L^{-1} L-cysteine, 3 mM MES, 3 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, 100 µM acetosyringone and 1 mM DTT in 0.8% (w:v) agar, pH 5.5). Explants were incubated for 5 days then washed twice (10 min each) in sterile distilled water with shaking at 100 rpm and blotted on filter paper. They were then transferred to fresh 100cm diameter x 25cm deep sized round Petri dishes containing regeneration medium (3.2 g L^{-1} SH salts, 1.01 g L^{-1} SH vitamins, 20 g L^{-1} sucrose, 3 mM MES, 1 mg mL^{-1} BAP, 1 mg mL^{-1} NAA, 10 mg mL^{-1} $AgNO_3$, 100 mg mL^{-1} cefotaxime and 500 mg mL^{-1} ticarcillin in 0.8% (w:v) agar, pH 5.8) and incubated for 15 days. Explants were then transferred to selection medium - regeneration medium supplemented with 1.6 mg L^{-1} phosphinothricin (PPT). They were transferred to fresh medium every 2 weeks and any explants not regenerating shoots were removed. Shoots developed from the explants were transferred to Phytatrays (Sigma-Aldrich) containing plant development and rooting medium (3.2 g L^{-1} SH salts, 1.01 g L^{-1} SH vitamins, 10 g L^{-1} sucrose, 0.5 mg mL^{-1} IBA, 100 mg mL^{-1} cefotaxime and 500 mg mL^{-1} ticarcillin in 0.25% (w:v) phytagel, pH 5.8). Plantlets with well-developed roots after 1 month were transplanted onto a soil mixture of 1 part Levingtons F2s, 1 part Vermiculite plus Osmocote added at 3 gm per liter in the glasshouse regulated to 22 ºC with a 16/8 hr light/dark photoperiod. The pots were covered with plastic lids for 3-4 days to maintain high humidity. The

plants were kept in the glasshouse for almost 3 months until most seedpods were developed. Then watering of the plants was stopped to induce completion of the set of mature seeds. Seedpods were harvested after they were fully dried and kept in paper bags at room temperature in laboratory conditions.

The T1 plantlets from the seeds were tested for the insertion of GFP. For this nine seeds grown as in section 2.2. DNA from young leaflets (1 week old) was extracted (as in section 2.5.1.2) and PCR was performed to identify the presence of GFP in the plants. The primer used for control samples was: forward primer 5'CACCAATCAAACTTCTTTTTTC and reverse primer 5'TGCTATTGCTAATGTGTTTCTC. GFP primers were: forward primer 5'CGCACCATCTTCTTCAAGGAC and reverse primer 5'AACTCCAGCAGGACCATGTGA. The GFP positive plants were transferred to soil and T2s were generated from them.

Chapter 3

Results: Phenotypic crosstalk between nodulation and lateral root development

Depending on the soil N concentration and form in the root environment, both rapid and inhibitory responses related to N uptake and assimilation is triggered in the plant. Inorganic and organic N have critical roles as signal molecules controlling genome-wide gene expression in response to N availability (Stitt, 1999, Redinbaugh and Campbell, 1991, Crawford, 1995). N acquisition is balanced through local and systemic signals that induce or repress LR formation according to the plant N and carbon status (Forde, 2002, Salon et al., 2009, Dechorgnat et al., 2011). Rapid responses caused by local and systemic signals during N limitation result in nodulation and root architecture development and branching (Jeudy et al., 2010, Salon et al., 2009).

The three dimensional structure of the root known as the RSA comprises of PR, LRs and root hairs. RSA is a highly plastic trait. RSA is one of the first contacting point with N and it is under the influence of external N in its environment and the plant internal N status thus it could be modulated by promotion or inhibition of PR and LR growth in response to N. N effect on RSA depends on several factors such as N source (Patterson et al., 2010), concentration (Wang et al., 2007), tissue type (Wang et al., 2003), cell type (Gifford et al., 2008) and time after treatment (Krouk et al., 2010b).

Phenotypic analysis of plant response to N in the presence of rhizobia was used to study the changes in root architecture when *Medicago* plants were subjected to a range of low and high NH_4NO_3 regimes as source of N and hence will be referred to here in this thesis as N. NH_4NO_3 was selected as N source based on similar experiments on *Arabidopsis* (Gifford,

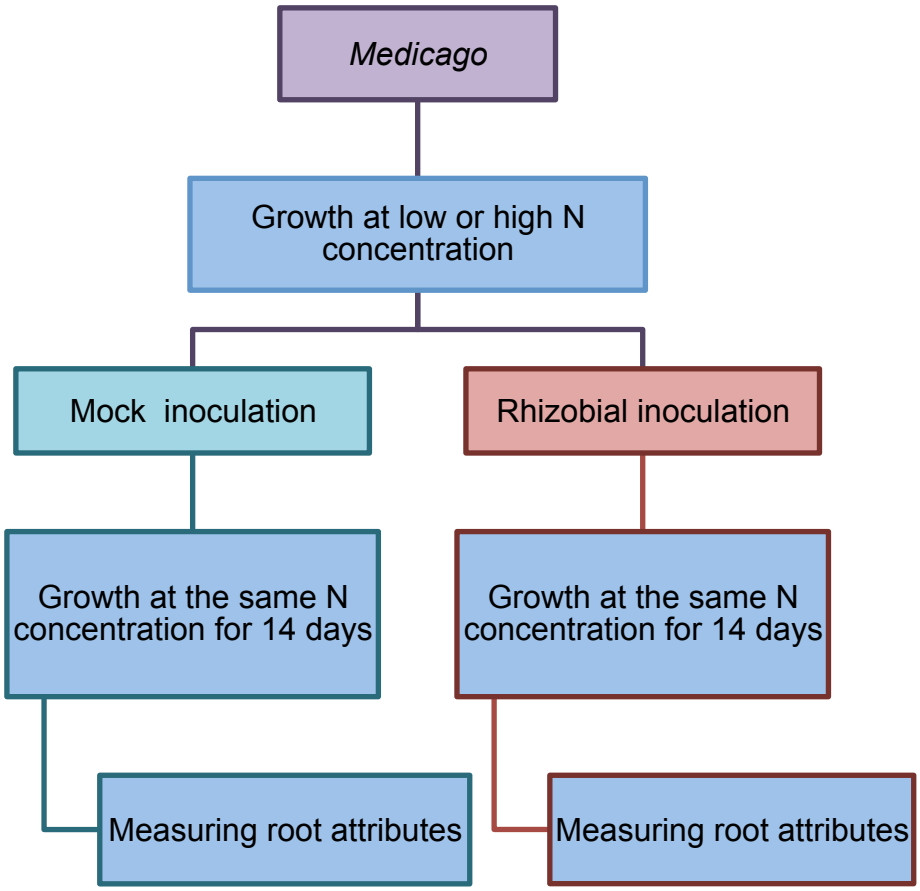


Figure 3-1: Experimental design for studying the effect of low and high N and rhizobia inoculation on RSA.

unpublished results) and studies using NH_4NO_3 for high N treatment as a more sufficient N source than NH_4 or NO_3 (Jeudy et al., 2010, Krouk et al., 2006).

3.1 Characterizing root architecture changes under low and high N concentrations and rhizobial inoculation

To study how nodulation affects PR and LR development under limiting and excessive N concentrations, a series of phenotyping experiments were carried out on *M. truncatula*. Seedlings were inoculated with rhizobia (+Rhiz) or mock inoculated (-Rhiz) to see how the ability to nodulate or the presence and absence of rhizobia can affect RSA under low and high N availability (Figure 3-1). RSA was quantified at 14 dpi by measuring PR length, LR total length, average of LR length, LR number, LR density, total root size and nodule number (Figure 2-2). This time point was chosen based on preliminary experiments (Gifford, Unpublished results) and pervious studies (Jeudy et al., 2010) showing that nodule development was more advanced at this point and a clearer comparison between -Rhiz and +Rhiz plants were possible.

Each data set was then transformed as log10 (y + a) or $\sqrt{}$ (y + a), where 'y' was the raw data and 'a' the min value in each data set and 0.5 for number of nodules. The transformation that approximately followed a normal distribution was selected for further analysis. This was done by plotting the histogram of residual values, normal plot and half normal plot using GenStat software (VSNInternational, 2011). REML (P<0.05) and the least significant difference (LSD) at 5% level were calculated for the effects showing significance (P<0.05) using GenStat.

3.1.1 Response to N and rhizobia in different *M. truncatula* ecotypes

In order to see if different *Medicago* genetic backgrounds show the same response trend to low and high N concentrations and rhizobia inoculation A17 and four other ecotypes of *Medicago* (cv. Jemalong 2HA, ssp. *tribuloides*, Gaertner and var. longispina) were grown at low (0.5 and 1 mM)

Table 3-1: REML *P* values from analysis of the differences between trait values in rhizobia inoculated and mock inoculated at low (0.5 and 1 mM) and high (2 and 10 mM) concentrations of N in *M. truncatula* var. Jemalong A17, var. Jemalong 2HA, Gaertner, var. longispina and ssp. *tribuloides*. PR= primary root and LR= lateral root.

Effect of low and high N concentrations and rhizobia on root system architecture of *Medicago* ecotypes							
	F pr						
	PR length	**Average of LR length**	**LR total length**	**LR density**	**Total root size**	**LR number**	**Nodule number**
Genotype	<0.001*	<0.001*	<0.001*	0.285	<0.001*	<0.001*	<0.001*
N concentration	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Rhizobia	0.003*	0.391	0.741	0.167	0.063	0.657	
Genotype x N concentration	<0.001*	<0.001*	<0.001*	0.005*	<0.001*	<0.001*	0.001*
Genotype x rhizobia	0.559	0.089	0.592	0.876	0.586	0.662	
N concentration x rhizobia	0.263	0.257	0.433	0.689	0.5	0.583	
Genotype x N concentration x rhizobia	0.21	0.002*	0.012*	0.102	0.015*	0.082	

* $P < 0.05$ in REML variance components analysis

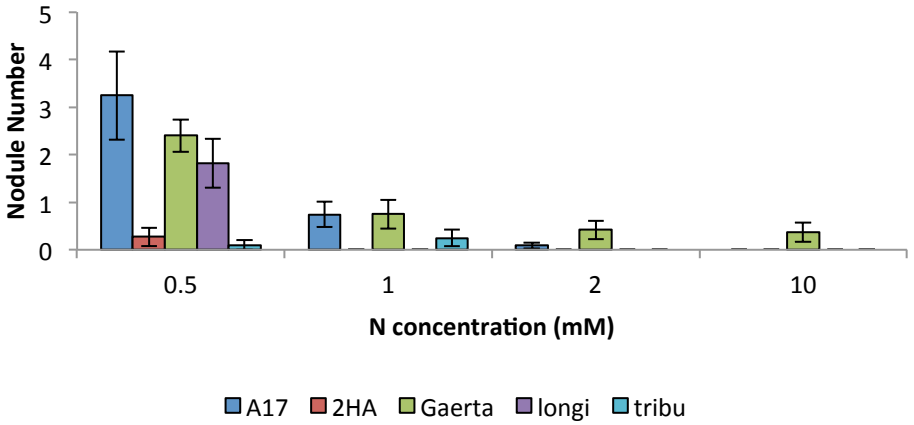


Figure 3-2: Effect of low (0.5 and 1) and high (2 and 10 mM) N concentrations on nodulation of *M. truncatula* var. Jemalong A17, var. Jemalong 2HA, Gaertner, var. longispina and ssp. *tribuloides* at 14 dpi with rhizobia. Bars represent the mean of 2 replicates (n=6 plants per replicate) +/- the standard error.

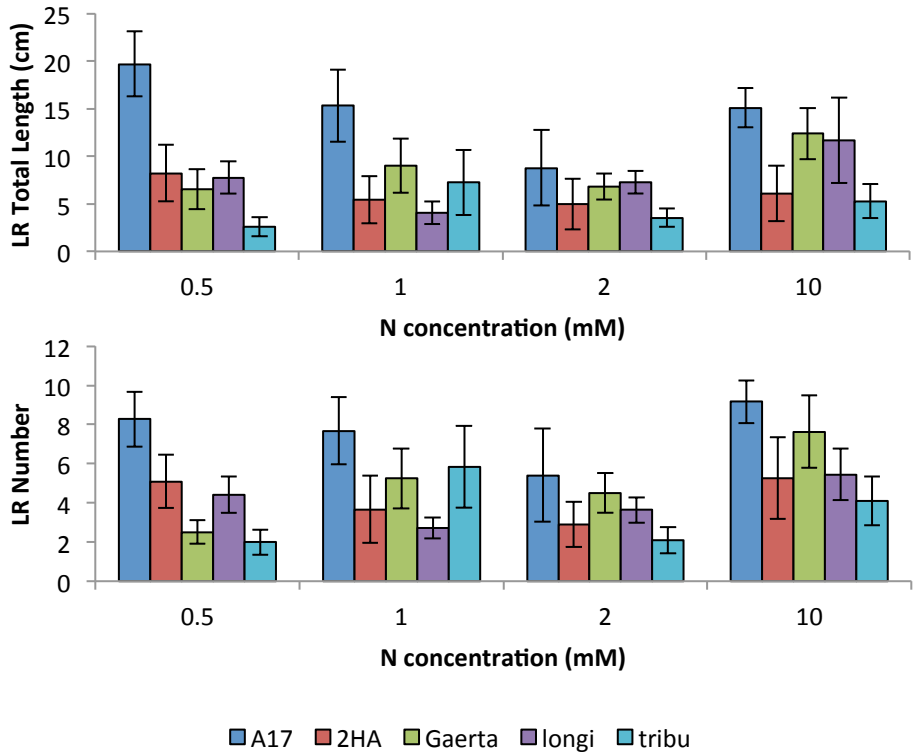


Figure 3-3: A comparison between the response of *M. truncatula* (a) var. Jemalong A17 with (b) var. Jemalong 2HA, (c) Gaertner, (d) var. longispina and (e) ssp. *tribuloides* to low (0.5 mM) and high (10 mM) N at 14 dpi with rhizobia. Bars represent the mean of 2 replicates (n=6 plants per replicate) +/- the standard error.

and high (2 and 10 mM) N concentration and inoculated or mock inoculated with rhizobia (Figure 3-1). The low and high concentrations of N were selected based on the N concentrations used at similar experiments on *Medicago* and *Arabidopsis* (Gifford, Unpublished results) and previous published studies (Jeudy et al., 2010). Samples were in 2 biological replicates (n=6 plant per replicate). Root attributes were measured at 14 dpi. REML analysis was used to identify significant (P<0.05) differences between mean values of the measured root attributes affected by the genotype, N concentrations and rhizobia.

The study showed that the measured root attributes (PR length, average of LR length, LR total length, LR density, total root size, LR number and nodule number) were significantly (P<0.05) affected by N concentration and the interaction between genotype and N concentration (Table 3-1 and figure 3-2). Genotype also had a significant effect on PR length, average of LR length, LR total length, total root size, LR number and nodule number. The interaction between genotype, N concentration and rhizobia significantly affected LR length (LR total length and average of LR length) and root total size (Table 3-1). Although the effect of genotype was significant (P<0.05), the aim of this experiment was to study the overall response trend of these genotypes to low and high N not to compare the response of individual genotypes with each other. The results suggests that -Rhiz and +Rhiz plants of A17 and the ecotypes used in this study showed a similar response trend to increasing concentrations N (Figure 3-3) irrespective of their individual differences regarding changes in root architecture. This could suggest that the N effect is conserved across ecotypes. Thus, further experiments were focused on A17 and the hypernodulating mutant, *sunn-1.*

3.1.2 Effect of low and high N concentration and rhizobia inoculation on RSA

To study the effect of low and high N concentration and rhizobia inoculation on RSA mock or rhizobia inoculated seedlings of *M. truncatula*

Table 3-2: REML *P* values from analysis of the differences between trait values in rhizobia inoculated and mock inoculated A17 plants at low and high concentrations of N (ranging from 0-20 mM). PR= primary root and LR= lateral root.

Effect of N concentration (0-20 mM) and rhizobia on A17 root system architecture							
	F pr						
	PR length	Average of LR length	LR total length	LR density	Total root size	LR number	Nodule number
N concentration	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Rhizobia	0.01*	0.025*	0.4	0.127	0.107	0.951	
N concentration x rhizobia	0.002*	0.359	0.642	0.882	0.089	0.527	

* *P* < 0.05 in REML variance components analysis

Figure 3-4, (a)

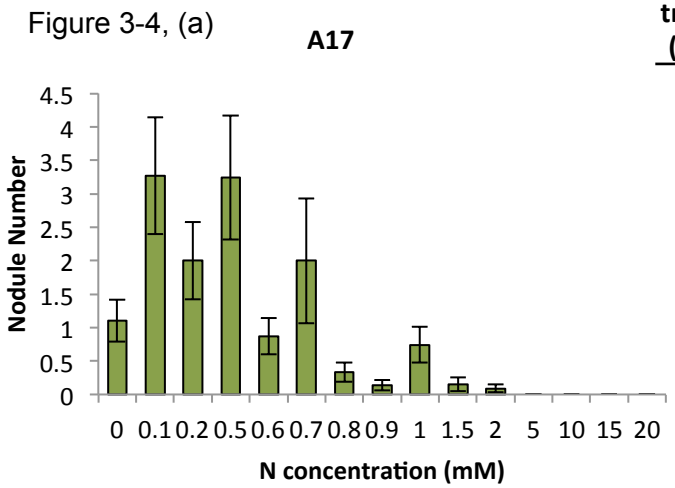
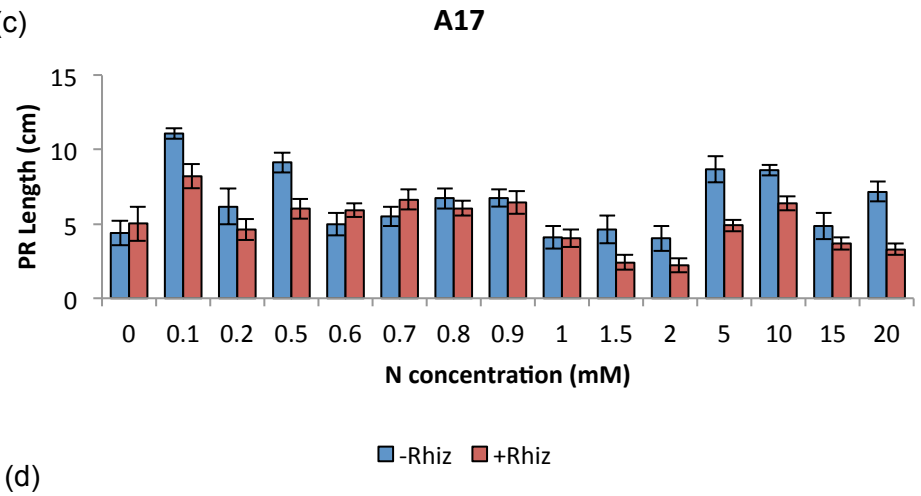


Figure 3-4, (b)

N effect			
N concentration (mM)	Mean (y)	Mean log10 (y +0.5)	LSD (5%)
0	1.10	0.11	0.26
0.1	3.27	0.44	
0.2	2.00	0.27	
0.5	3.57	0.28	0.16
0.6	0.77	-0.05	
0.7	1.83	0.01	0.17
0.8	0.31	-0.18	
0.9	0.14	-0.24	
1	0.74	-0.10	
1.5	0.15	-0.23	
2	0.09	-0.26	
5	0.00	-0.30	
10	0.00	-0.30	
15	0.00	-0.30	
20	0.00	-0.30	

Max LSD = 0.27

(c)



(d)

N and rhizobia effect on PR length							
	-Rhiz		+Rhiz		N effect at -Rhiz	N effect at +Rhiz	Rhizobia effect
N concentration (mM)	Mean (y)	Mean log10(y+a)	Mean (y)	Mean log10(y+a)	LSD (5%)	LSD (5%)	LSD (5%)
0	4.39	0.62	5.01	0.60	0.28	0.26	
0.1	11.08	1.05	8.23	0.89	0.26		
0.2	6.18	0.71	4.63	0.63	0.23		
0.5	9.55	0.96	6.53	0.70	0.18		0.17
0.6	4.98	0.53	5.93	0.73			0.17
0.7	5.49	0.66	6.63	0.75			
0.8	6.71	0.75	6.06	0.75			
0.9	6.76	0.76	6.43	0.70	0.16	0.17	
1	4.12	0.39	4.02	0.44			
1.5	4.66	0.52	2.41	0.30			
2	4.02	0.46	2.21	0.23	0.23	0.21	0.19
5	8.69	0.91	4.89	0.69			
10	8.62	0.93	6.37	0.79	0.23	0.22	
15	4.86	0.63	3.66	0.56			
20	7.18	0.85	3.30	0.51			0.27

Max LSD = 0.29

Figure 3-4: Effect of N concentration (ranging from 0 to 20 mM) and rhizobia on A17 (a) nodule number and (c) primary rot (PR) length at 14 dpi. Significance (P<0.05) are shown on tables b (for nodule number) and d (for PR length). In the graphs bars represent the mean of raw data (2 replicates, n=6 plants per replicate) +/- the standard error. In the tables: y= raw data, a=min value in the data set. The LSD values are written only where there is significance (P<0.05) between two consecutive N concentrations (N effect) or between -Rhiz and +Rhiz (rhizobia effect).

A17 were grown at 0, 0.1, 0.2, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 5, 10, 15 and 20 mM concentrations of N (Figure 3-1). At 14 dpi different root attributes of 2 biological replicates (n=6 plant per replicate) were measured. The data was analysed using REML to identify the effects (N concentration and rhizobial treatment) that were significantly (P<0.05) effecting RSA changes.

The results showed that N concentration had affected PR and LR development and nodule formation (Table 3-2). It significantly (P<0.05) affected all the measured root attributes (PR length, average of LR length, LR total length, LR density, total root size, LR number and nodule number) and rhizobial treatment had significant (P<0.05) effect on PR length and average of LR length (Table 3-2).

N concentration affected the ability of the plants to nodulate. Nodule development decreased with the increase in N concentration (Figure 3-4 a and b). This N response is in agreement with previous studies (Streeter and Salrainen, 1988, Charon et al., 1999, Glyan'ko et al., 2009). Nodulation was observed in seedlings grown on concentrations of N from 0 to 2 mM and inhibited by high (starting from 5 mM) concentrations of N. A larger number of nodules were generally observed at the lower concentrations (0 to 0.5 mM) with the highest number of nodulation at 0.1 and 0.5 mM and then declined (Figure 3-4 a and b). The formation of new nodules is inhibited at high concentrations of N (Streeter, 1985, Thimm et al., 2004) and N fixation capacity of existing nodules is also affected by high N (Cabeza et al., 2014). As nodule development is a costly process for the plant , when NH_4^+ and NO_3^- are available, it is more favored by the plant to uptake these ions rather than use the costly produced N_2 in the nodules (Silsbury et al., 1986, Wery et al., 1986, Carroll et al., 1990).

The interaction between N concentration and rhizobia inoculation had a significant (P<0.05) effect on PR length (Table 3-2). Low and high N concentrations in both -Rhiz and +Rhiz significantly (P<0.05) affected PR length (Figure 3-4 c and d). Although PRs are less sensitive to nitrate than

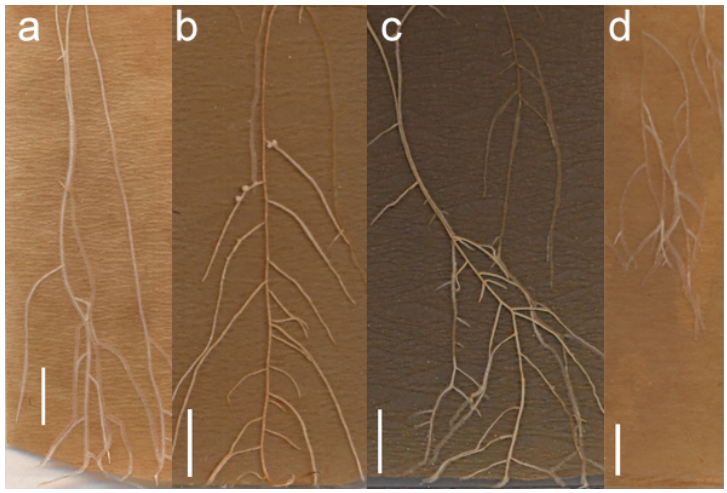
LRs, studies shows that they could be affected by nitrate concentration (Forde, 2014, Vidal et al., 2013). Rhizobia also had a significant (P<0.05) effect on PR growth at low and high N concentrations (Figure 3-4 c and d).

This study was also used for selecting low and high concentrations of N for further studies on the effect of low and high N concentration and rhizobia on root development and nodule formation. Plants grown on 1 to 0.5 mM of N produced a higher number of nodules. Since the results obtained from REML showed that there was no significant difference (P<0.05) between these concentrations (as the low range), hence 0.1 mM of N was chosen as the low N concentration treatment and 5 mM N, the concentration that nodulation was inhibited, as high N. These concentrations were also the same as the N concentrations used for studies on N response in *Arabidopsis thaliana* (Gifford, unpublished results) and selecting the similar concentrations made the comparison of the experiments possible. Thus unless mentioned otherwise, low and high concentrations of N are 0.1 and 5 mM respectively.

3.1.2.1 Rhizobia effect on root development

To study the effect of rhizobia at low (0.1 mM) and high (5 mM) N concentration on LR and PR development, mean values of -Rhiz and +Rhiz samples at 0.1 and 5 mM N were compared together using a two sample Student's t-test at 5% significance level (Figure 3-5 e). The results from Student's t-test were also confirmed by REML analysis of the data (results not shown).

At low N Nodulation or the presence of rhizobia had no effect on LR development (Figure 3-5 e) and no significant difference was observed in the average of LR length, LR total length, LR number and LR density between -Rhiz and +Rhiz (Figure 3-5 e). The results showed that rhizobia affected root development at high N concentration even though nodulation was inhibited. This was suggested because of the significant (P<0.05) effect of rhizobia on PR growth, LR total length, LR number and root total size (Figure 3-5). There is evidence that symbiotic organisms can affect LR development



(e)

Rhizobia effect						
N concentration (mM)	**PR length**	**Average of LR length**	**LR total length**	**Total root size**	**LR density**	**LR number**
0.1	0.0038*	0.5164	0.8705	0.5130	0.6299	0.9010
5	0.0004*	0.9020	0.0050*	0.0015*	0.1355	0.0035*

Low and high N effect							
N concentration (mM)	**PR length**	**Average of LR length**	**LR total length**	**Total root size**	**LR density**	**LR number**	**Nodule number**
0.1 to 5 at -Rhiz	0.0007*	0.7413	0.2409	0.0585*	0.1199	0.1974	0.0003*
0.1 to 5 at +Rhiz	0.0203*	0.8155	0.0469*	0.2272	0.0026*	0.0560*	

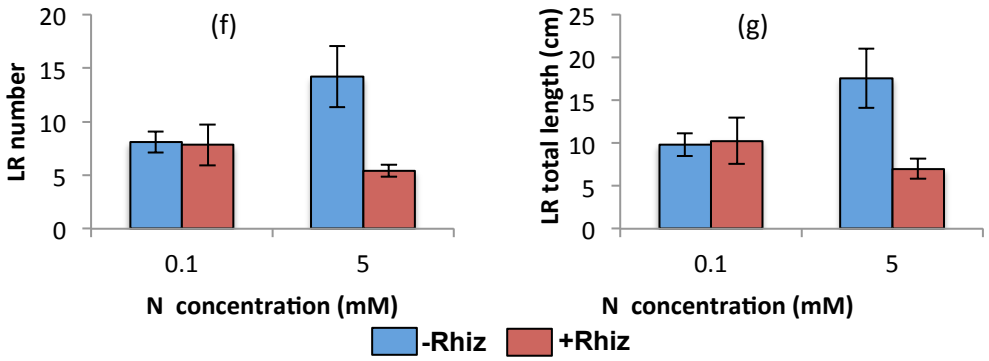


Figure 3-5: Rhizobia affects root development at high N concentration where nodulation is inhibited. (a-d) shows the effect of a, b) low (0.1 mM) and c, d) high (5 mM) N concentrations on A17 root architecture at 14 dpi with rhizobia in mock inoculated (a and c) and rhizobia inoculated(b and d). Scale bar = 10mm. (e) t-test *P* values from analysis of the differences between mean of trait values in either rhizobia inoculated (+Rhiz) vs mock inoculated (-Rhiz), or low vs high N in A17. Asterisks = *P*<0.05 (f) LR total length and (g) number of LRs. Bars represent the mean of 2 replicates (n=6 plants per replicate) +/- the standard error. PR= primary root and LR= lateral root.

(Oláh et al., 2005, Maillet et al., 2011). In *Medicago*, LR formation could be stimulated by Nod factors. This stimulation requires NFP, DMI1, DMI2, DMI3 and NSP1 that are also involved in the symbiotic pathway (Oláh et al., 2005). This suggests that nodulation or Nod factors could interfere with the root developmental programme. At high N, total root size was significantly (P<0.05) less in +Rhiz compared to -Rhiz due to significantly (P<0.05) shorter PRs (Figure 3-4 c) and significantly (P<0.05) smaller LR total length and number (Figure 3-5) compared to -Rhiz. Flavonoids that also act as signal molecules in the legume symbiosis pathway, could effect LR development. The accumulation of flavonoids decreases polar auxin transport leading to short LRs (Laffont et al., 2010). LR development is also regulated through local and systemic pathways depending on the plant N status (Remans et al., 2006a, Desnos, 2008, Forde, 2014) including repression of root development by products of N assimilation (Gifford et al., 2008). In *Arabidopsis* low concentrations of glutamate (<50 mM) had an inhibitory effect on PR growth and stimulated LR outgrowth that resulted in shorter and more branched LRs (Forde, 2014). Studies on *Arabidopsis* also shows that PR growth could be inhibited by application of high (5 mM) nitrate which was associated with increase in auxin concentration at the root tip (Vidal et al., 2010).

3.2 Studying the timescale of LR and nodule development

The phenotypic analysis provided a long-term perspective of the timescale of LR development and nodulation. We were interested to see how N treatment and rhizobial inoculation changed the number of LRs and their stages of development and the timescale on which these changes occurred.

Different stages of LR development and patterning have been studied in detail in *Arabidopsis thaliana* (Malamy and Benfey, 1997, Lucas et al., 2013, Péret et al., 2013). In a recent study using DR5:GUS and LaS-CARECROW:GUS (LaSCR:GUS) marker genes cellular events of LR formation was studied in *M. truncatula* (Herrbach et al., 2013). In this study 7 stages were defined for LR development (Figure 3-6). In the first stage (stage

Ia) two or three pericycle cells go into anticlinal division (Figure 3-6 A). In stage Ib this anticlinal division in pericycle continues and at the same time endodermis cells start dividing anticlinally (Figure 3-6 B). The anticlinal division of endodermis continues in stage II and in the pericycle two layers are formed through periclinal division (Figure 3-6 C). Cells in pericycle and endodermis divide periclinally to form a four-layered pericycle and two-layered endodermis in stage III and cells in inner cortex also start anticlinal division (Figure 3-6 D). These divisions in pericycle, endodermis and inner cortex continues in stage IV (Figure 3-6 E). In this stage anticlinal divisions of the inner cortex forms the external part of LR primordia and proliferation of the parenchyma forms the future vasculature. In stage V LRP is expanded by cell proliferation in pericycle, endodermis and inner cortex layers (Figure 3-6 F). In stage VI LRP reaches the epidermis but has not merged from this cell layer (Figure 3-6 G) and in stage VII (Figure 3-6 H) the fully developed LR emerges from epidermis. Based on these developmental stages, the time scale of LR development under low and high N treatments and rhizobial inoculation was studied (Figure 3-7).

Mock (-Rhiz) or rhizobia (+Rhiz) inoculated seedlings of A17 were grown at low (0.1) N concentration. At 14 dpi, -Rhiz and +Rhiz samples were treated with either low or high N and a group of -Rhiz was rhizobia inoculated and treated with low N (Figure 3-7). The samples were transferred to medium containing the same N concentration as treatment and roots in three replicates (n=3) were harvested at 0, 4, 8, 12 and 16-day time points and viewed under the microscope. The data were then transformed using logarithmic or square root transformation (as explained in section 3.1) and analysed using REML to identify the effect of rhizobia at low and high N and the effect of rhizobia inoculation at low N over the 16-day time points (Figure 3-6).

Four stages were defined for LR development based on the stages identified by (Herrbach et al., 2013): stage i of initiating LRP (LRP i): stages I-III (in Figure 3-6 A-D), stage ii of initiating LRP (LRP ii): stages IV-V (in Figure

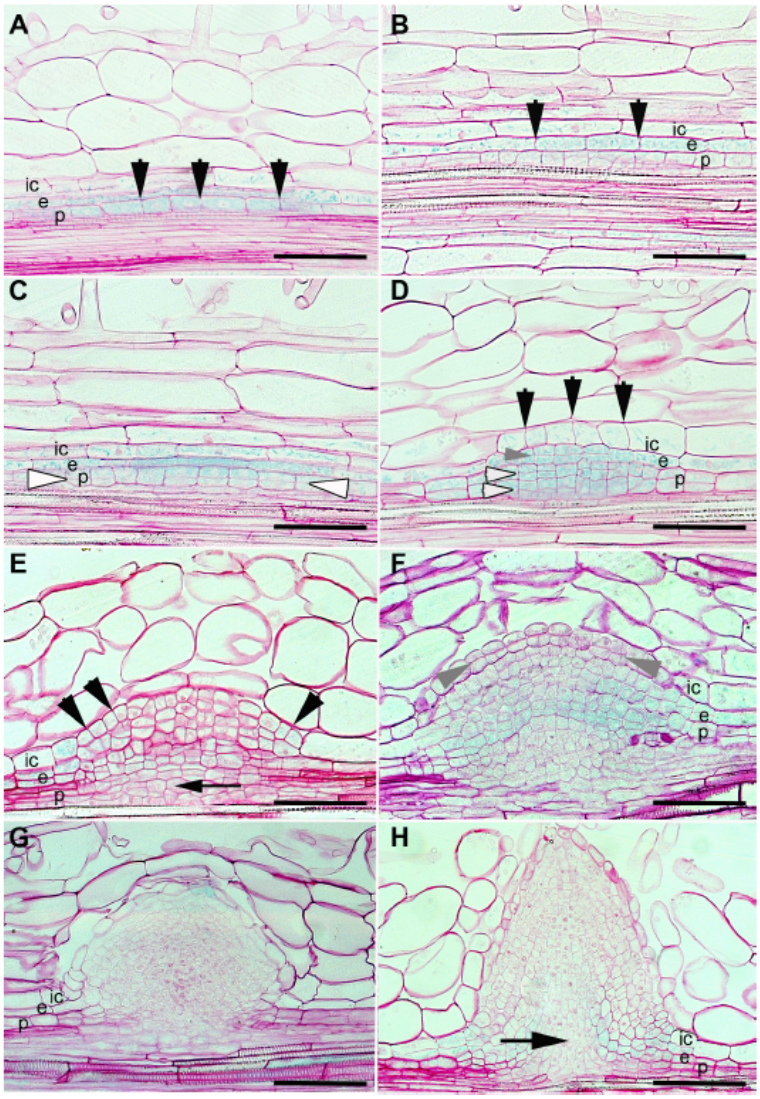


Figure 3-6: Developmental stages of lateral root formation in *M. truncatula*. (A) Stage Ia: Anticlinal divisions in the pericycle (black arrowheads). (B) Stage Ib: Anticlinal division in pericycle cells and epidermis (black arrowhead). (C) Stage II: A two-layer pericycle is formed through periclinal division (white arrowheads) and the endodermis continues its anticlinal division. (D) Stage III: Formation of a four-layered pericycle (white arrowheads) and two-layered endodermis (grey arrowhead) by periclinal divisions, and anticlinal divisions in the inner cortex (black arrowheads). (E) Stage IV: Divisions in the pericycle, endodermis and inner cortex continues. Anticlinal division of inner cortex forms the external part of LRP (black arrowhead) and proliferation of the parenchyma (arrow) forms the future vasculature. (F) Stage V: expansion of the LRP by pericycle, endodermis and inner cortex cells proliferation. The grey arrowhead shows periclinal divisions in the most external layer of the LRP. (G) Stage VI: LRP reaches the epidermis and is about to emerge. (H) Stage VII: Emergence of the LR. The arrow shows the central vasculature of the new root with many elongated cells. Bars = 100 μm. (Herrbach et al., 2013)

3-6 E-F), emerging LR: stage VI (in Figure 3-6 G), fully emerged LR: stage VII (in Figure 3-6 H). Nodule primordium, emerging nodule and fully developed nodule was defined as in (Kuppusamy et al., 2009) (Figure 3-8). In their study, Kuppusamy et al. (2009) reported that MtCDC16 was involved in controlling the number of LRs and nodules in *M. truncatula*. The expression of MtCDC16 was activated in cell division zone in growing roots and nodules of *M. truncatula* (Kuppusamy et al., 2009). To study the expression pattern of MtCDC16 the putative promoter region of MtCDC16 was fused to a GUS reporter gene and the transcriptional activation of the reporter gene was monitored. In response to rhizobia, GUS expression was observed in nodule primordia, emerging nodules and finally was limited to the nodule meristem of the fully developed nodule (Figure 3-8) (Kuppusamy et al., 2009).

REML analysis of -Rhiz and +Rhiz samples treated with low N (A-D in Figure 3-7) showed that rhizobia had no significant effect on different stages of LR development at low N (Table 3-3). A significant (P<0.05) rhizobia effect was observed in sample treated with high N (B-E in Figure 3-7) on early and late stages of LR development (LRP i and fully emerged LRs) (Table 3-3). The rhizobia effect on different stages of LR development at low and high N was consistent with the results obtained from section 3.1.2.1. Studying different stages of LR development over the 16-day period post treatment of -Rhiz samples at low N with rhizobia (A-C in Figure 3-7) showed that rhizobia inoculation had significant (P<0.05) effect on the early stages of LR development (stages i and ii of LRP development) (Table 3-3).

A two sample Student's t-test at 5% significance level was used to compare the mean values of different LR developmental stages in -Rhiz with +Rhiz samples at low N (A and D in Figure 3-7), -Rhiz with +Rhiz at high N (B and E in Figure 3-7) and -Rhiz at low N with -Rhiz treated with rhizobia at low N (A and C in Figure 3-7) to identify the significant effect of rhizobia at these different conditions and over different time points (Figure 3-9). The results of the t-test analysis were also confirmed by the REML analysis (Table 3-3).

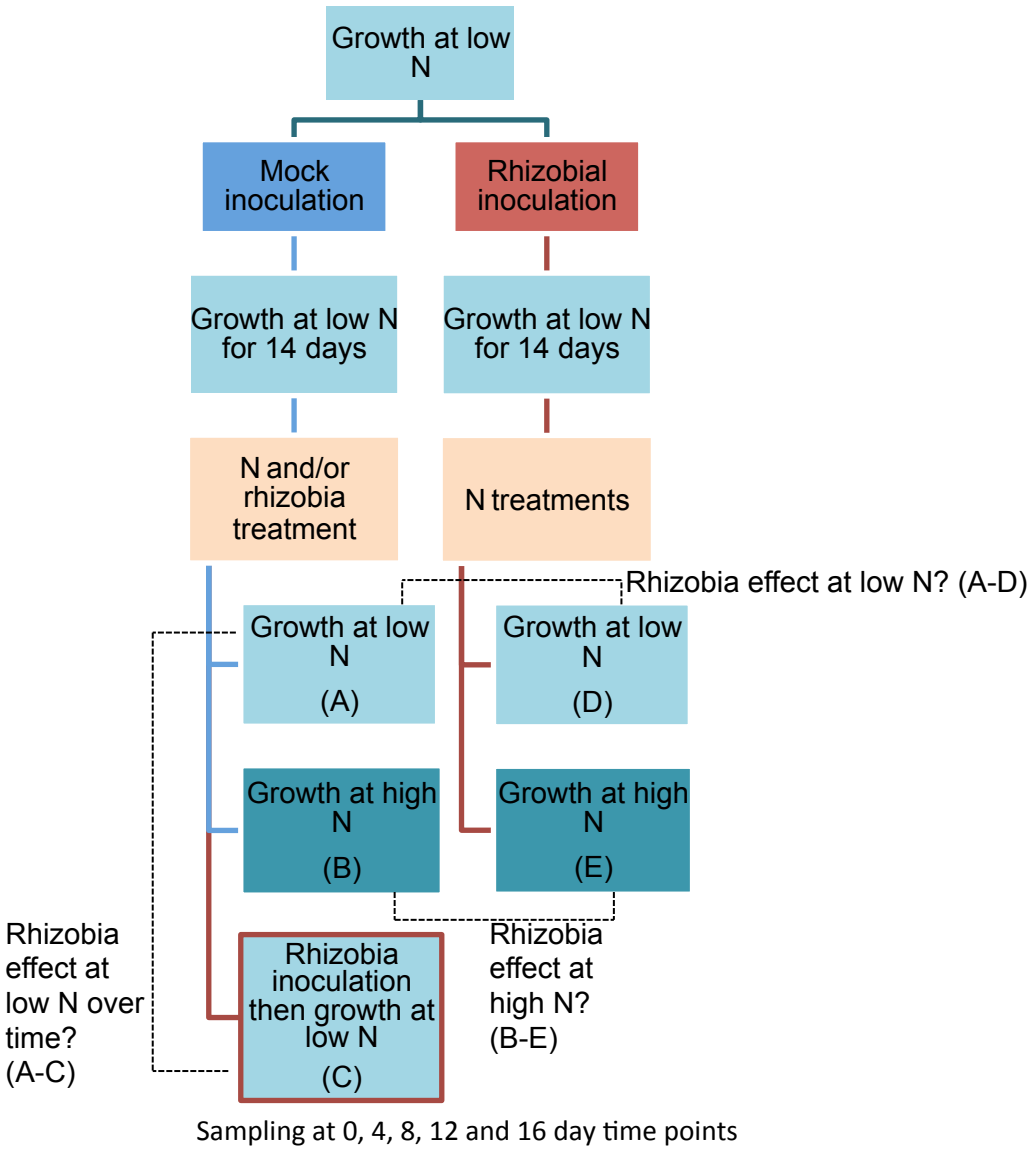


Figure 3-7: Diagram showing treatments applied to A17 plants for studying the timescale of LR and nodule development under low and high N concentrations.

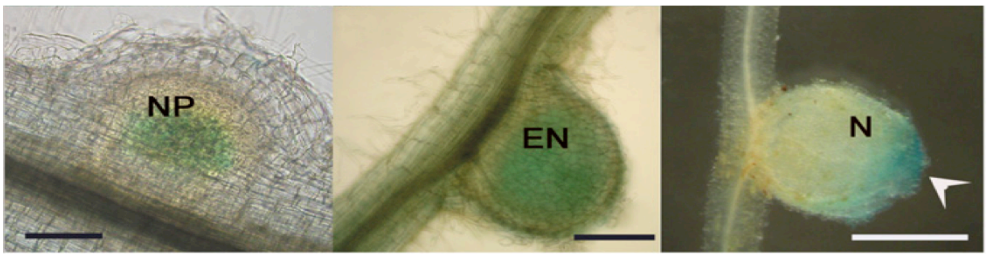


Figure 3-8: Nodule primordium (NP), emerging (EN) and fully developed (N) nodule in *Medicago truncatula* (Kuppusamy et al., 2009). Blue staining indicates GUS induction during nodule development. Bars = 50 mm.

At low N and in the absence of rhizobia (A in Figure 3-7) there was an initial increase in different stages of LR development (LRP i and ii, emerging and fully emerged LR) at 4-day time point, which was followed, by a decreasing trend at later time points (Figure 3-9 a). In response to N limitation, HATS is upregulated and LR development is stimulated. In WT plants of *Arabidopsis* when transferred from high to low nitrate concentration, the number of visible LRs increased under a moderate N limitation (transfer from 10 mM to low 1-0.5 mM) (Remans et al., 2006b). This response is followed by decrease in LR development that is controlled by local and systemic signaling pathways (Forde, 2014). In +Rhiz samples (D in Figure 3-7) there was an initial increase in LR development (LRP i, emerging LR and fully emerged LR) and as the number of nodules (nodule primordia and fully developed nodule) increased, no significant changes were observed in the number of LRs at different stages (Figure 3-9 a). These results show that how the LR developmental response to N is regulated by the local and systemic pathways that act based on the plant N status. It also shows how N deprived plants balance LR development with nodule formation.

To further study these responses, -Rhiz plants grown at low N condition were inoculated with rhizobia (C in Figure 3-7). The effect of nodulation or rhizobia was then studied on different stages of LR development at 0-16 days time points post inoculation. Rhizobia inoculation of plants showed that at early time points after inoculation where nodule primordia were forming, there was an increase in all stages of LR development (LRP i and ii, emerging and fully emerged LR) (Figure 3-9 c). At the early time points (4-8 days), the number of LRP i was significantly (P<0.05) less in +Rhiz compared to -Rhiz (Figure 3-9 c). This shows a rhizobia effect on LR development at the early stages. The number of LRs at different stages of development (LRP i and ii, emerging and fully emerged LR) decreased as the number of fully developed nodules increased (Figure 3-9 c) suggesting a balancing response between nodulation and LR development.

After growth at low N for 14 days, when -Rhiz plants were treated with

Table 3-3: REML *P* values from analysis of the differences between trait (different stages oflateral root (LR) development (Herrbach et al., 2013)) values in rhizobia inoculated and mock inoculated A17 plants treated with low or high N. LRPi and LRPii are stages i and ii of lateral root primordia (LRP) development respectively; dpt: days post treatment; (A-D), (B-E) and (A-C) correspond to the treatments and questions identified in figure 3-7.

		Rhizobia effect			
		LRPi	**LRPii**	**Emerging LR**	**Fully emerged LR**
Rhizobia effect at low N (A-D)	**Rhizobia**	0.765	0.368	0.202	0.184
	dpt	0.001*	0.776	0.864	0.343
	Rhizobia inoculation x dpt	0.129	0.408	0.984	0.145
Rhizobia effect at high N (B-E)	**Rhizobia**	0.036*	0.414	0.83	0.021*
	dpt	0.782	0.835	0.054*	0.051*
	Rhizobia x dpt	0.055*	0.214	0.124	0.714
Rhizobia effect at low N over time (A-C)	**Rhizobia**	<0.001*	0.005*	0.75	0.438
	dpt	0.007*	0.846	0.858	0.891
	Rhizobia x dpt	0.295	0.528	0.427	0.068

* $P < 0.05$ in REML variance components analysis

Figure 3-9

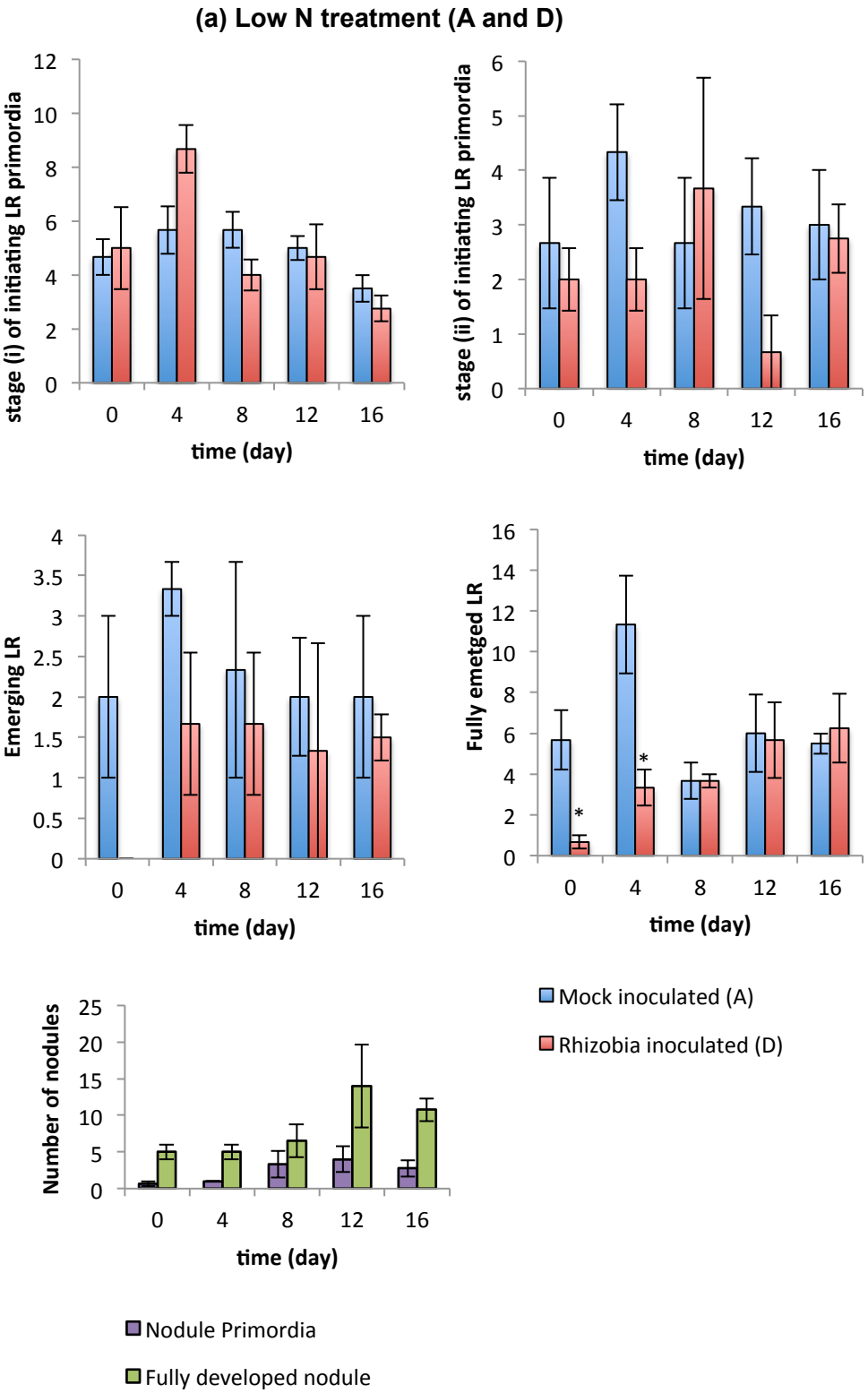
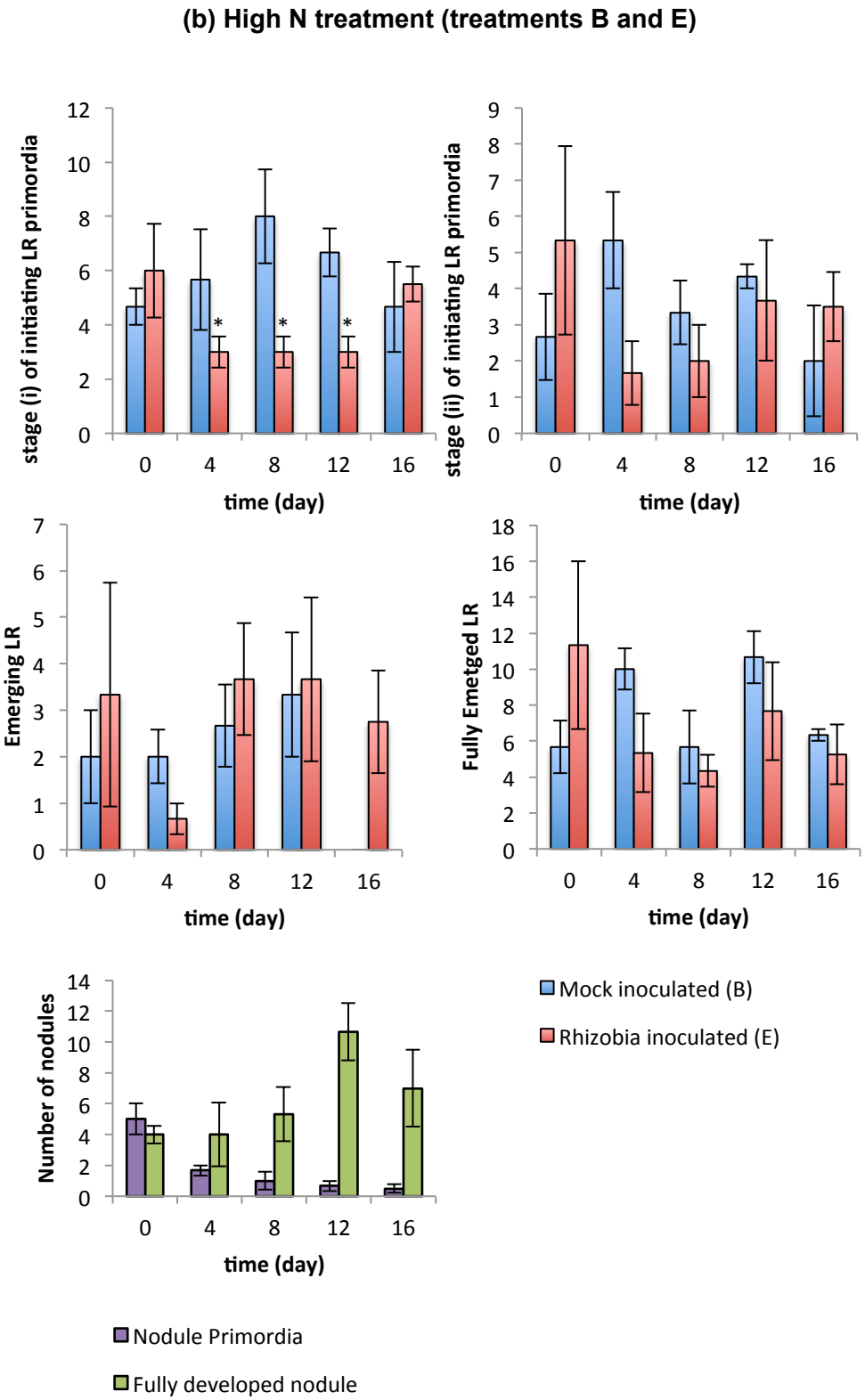

(a) Low N treatment (A and D)
stage (i) of initiating LR primordia
stage (ii) of initiating LR primordia
Emerging LR
Fully emetged LR
Number of nodules
time (day)
Mock inoculated (A)
Rhizobia inoculated (D)
Nodule Primordia
Fully developed nodule

Figure 3-9, continue



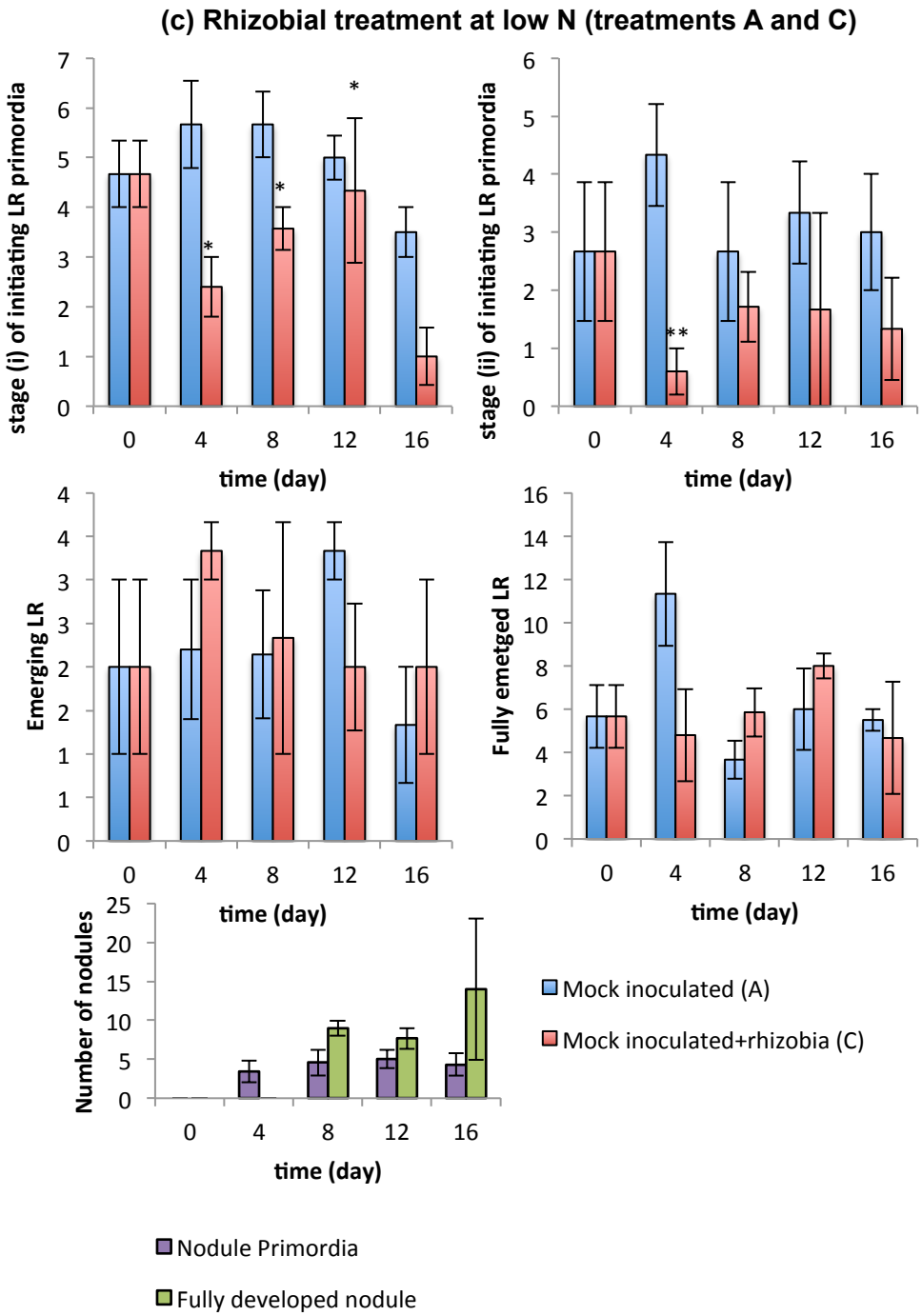


Figure 3-9: Effect of rhizobia on the time scale of LR development and nodule formation at (a) low N, (b) high N and (c) low N and rhizobia inoculated. Different stages of LR development was measured according to (Herrbach et al., 2013) over a 16 day time point. A, B, C, D and E correspond to treatments in figure 3-7. Significance testing: Bars represent the mean of three replicates, n=1 plant per replicate +/- the standard error. Asterisks above values for developmental stages at different time points denote significant differences between average values between the rhizobia-inoculated and mock-inoculated samples; * = $0.01<P<0.05$ and ** = $P<0.01$ in t-tests.

high N (B in Figure 3-7), there was an increasing trend in the number LRs in different stages of development (LRP i and ii, emerging and fully emerged LR) at the earlier time points that was followed by a decrease at the later time points (Figure 3-9 b). The decreasing trend at the later time points suggests a balancing response to high N according to the plant N status (Remans et al., 2006a, Desnos, 2008, Forde, 2014, Gifford et al., 2008). Nitrate acts locally by promoting LR growth in different developmental stages. This is an adaptive response enabling plants to respond to local patches of high nitrate in the soil. In split agar plate experiments in *Arabidopsis* the side of the root exposed to high nitrate showed preferential LR growth. LRP emergence was increased and root elongation was stimulated (Bouguyon et al., 2012). Studies in *Arabidopsis* show that high nitrate concentration (> 10 mM) inhibits LR growth after their emergence from the PR by affecting the activation of the LRP meristem. This results in short LRs whose elongation is repressed (Zhang et al., 1999). In +Rhiz (E in Figure 3-7) the number of LRs in stage i of LRP development were significantly (P<0.05) less than -Rhiz suggesting a balancing effect for rhizobia at high N (Figure 3-9 b). The number of nodule primordia decreased significantly (P<0.05) 4 days after treatment with high N and at the later time points where the number of nodule primordia decreased; there was an increasing trend in different stages of LR development (Figure 3-9 b).

3.3 Conclusion

The phenotypic study of root architecture changes in response to low (0.5 and 1 mM) and high (2 and 10 mM) N and rhizobia showed that this response was similar between A17 plants and the studied ecotypes (cv. Jemalong 2HA, ssp. *tribuloides*, Gaertner, longispina). In this study we also show that nodulation was inhibited at 5 mM and higher concentrations (up to 20 mM) of NH_4NO_3 as the source of N.

Studying the root architecture changes at low (0.1 mM) and high (5 mM) concentrations of N in -Rhiz and +Rhiz samples of A17 showed that the

rhizobia effect on PR and LR length and number was not significant at low N. The time point study of nodule and LR development at cellular level also was consistent with this result showing that rhizobia had no significant (P<0.05) effect on different stages of LR development (LRP i, LRP ii, emerging and fully emerged LRs) 0-16 days post treatment with low N. In the presence of rhizobia at low N there was an initial increasing trend in the number of LRs at different developmental stages (LRP i, emerging LR and fully emerged LR) but at the later time points no significant changes were observed. This trend in LR development and the increase in the number of nodule primordia and fully developed nodules at the later time points suggests a balance between LR development and nodule formation.

Studying the time scale of LR development and nodule formation 0-16 days post rhizobia inoculation of -Rhiz plants grown at low N shows that rhizobia had a significant (P<0.05) effect on the early stages of LR development (LRP i and ii). The number of LRP in stages i and ii was significantly (P<0.05) less in +Rhiz compared to -Rhiz. Also the number of LRs in different developmental stages decreased as the number of nodule primordia and mature nodules increased at the later time points. These all shows that how LR development and nodule formation are balance at low N.

Rhizobia significantly affected root development at high N even though at this concentration nodulation was inhibited. Total root size was significantly (P<0.05) less in the presence of rhizobia at high N. This was due to a significantly (P<0.05) shorter PR, smaller LR total length and less LR in number. High N treatment of N deprived plants also showed that rhizobia significantly (P<0.05) affected early and late stages of LR development (LRP i and fully emerged LRs). At early time points post treatment with high N there was an increasing trend in the number LRs in different stages of development (LRP i and ii, emerging and fully emerged LR) followed by a decrease at the later time points possibly caused by the signaling pathways involved in balancing LR development according to the plant N status.

The data obtained from these experiments formed a basis for a better understanding of how LR development and nodule formation or inhibition were balanced under low and high N concentrations and rhizobia inoculation. The effect of N concentration on RSA was significant in the presence of rhizobia. This effect was most interesting at high N (5 mM) where in the presence of rhizobia total root size was smaller (Figure 4-1) due to shorter PR and less number of LRs (average of LR length was not significantly different between treatments). In order to associate these phenotypic responses to gene expression changes for controlling LR development and nodulation, whole genome profiling experiments using microarrays was performed. The experiments were designed to study early (2 and 6 h) N-regulated responses to high N in the whole root. The rhizobia-mediated responses to low and high N concentrations were also studied. This enabled to (1) identify genes showing the strongest N and/or rhizobia regulated expression changes, (2) studying the gene expression patterns in different gene clusters and (3) identify the main biological pathways responsible for N-regulated and rhizobia-mediated responses of LR and nodule development. Since these responses are highly tissue specific, we also aimed to study the balance between LR and nodule development at cell specific level. These studies will be discussed in detail in chapter 4.

Chapter 4

Results: Expression analysis to identify genetic control of developmental crosstalk

Phenotypic study of RSA in response to low and high N and rhizobia showed that at high N concentration nodulation stops; there is a significant ($P<0.05$) decrease in root development in +Rhiz (smaller root size due to shorter PR and LRs and decrease in LR number compared to -Rhiz). This could suggest a balancing effect for rhizobia on root architecture development at high N or (Figure 4-1). To identify genes controlling these phenotypic effects, microarray experiments were designed to study the early responses (2 and 6 h) to high N (5 mM NH_4NO_3) treatment in rhizobia inoculated (+Rhiz) and mock inoculated (-Rhiz) A17 seedlings grown at low N (0.1 mM) concentration (Figure 4-2 a). Seedlings of +Rhiz or -Rhiz were grown at low N for 14 days and then treated with high N for 2 and 6 h (C2, C6, R2 and R6 in Figure 4-2 a) or harvested directly after growth at low N (C0 and R0 in Figure 4-2 a). In addition microarray experiments were designed to determine how gene expression changes when +Rhiz seedlings grown in high N were treated with low N (Figure 4-2 b). In this experiment +Rhiz A17 plants were grown on high N (5 mM) and at 14 dpi were treated with high (5 mM) or low N (0.1 mM) for 6 h (HH and HL respectively in Figure 4-2 b). The steps taken for analysing the microarray data and identifying significantly affected genes by rhizobia and N treatments are shown in Figure 4-2 c.

4.1 Quality control and normalization of the microarray data

Experimental conditions were selected to generate relevant data to answer specific biological questions about Rhizobial and N responses. Samples were prepared in 3 biological replicates and each biological replicate had 3 repeats (3 plates). Roots of 3 plants from each repeat were harvested

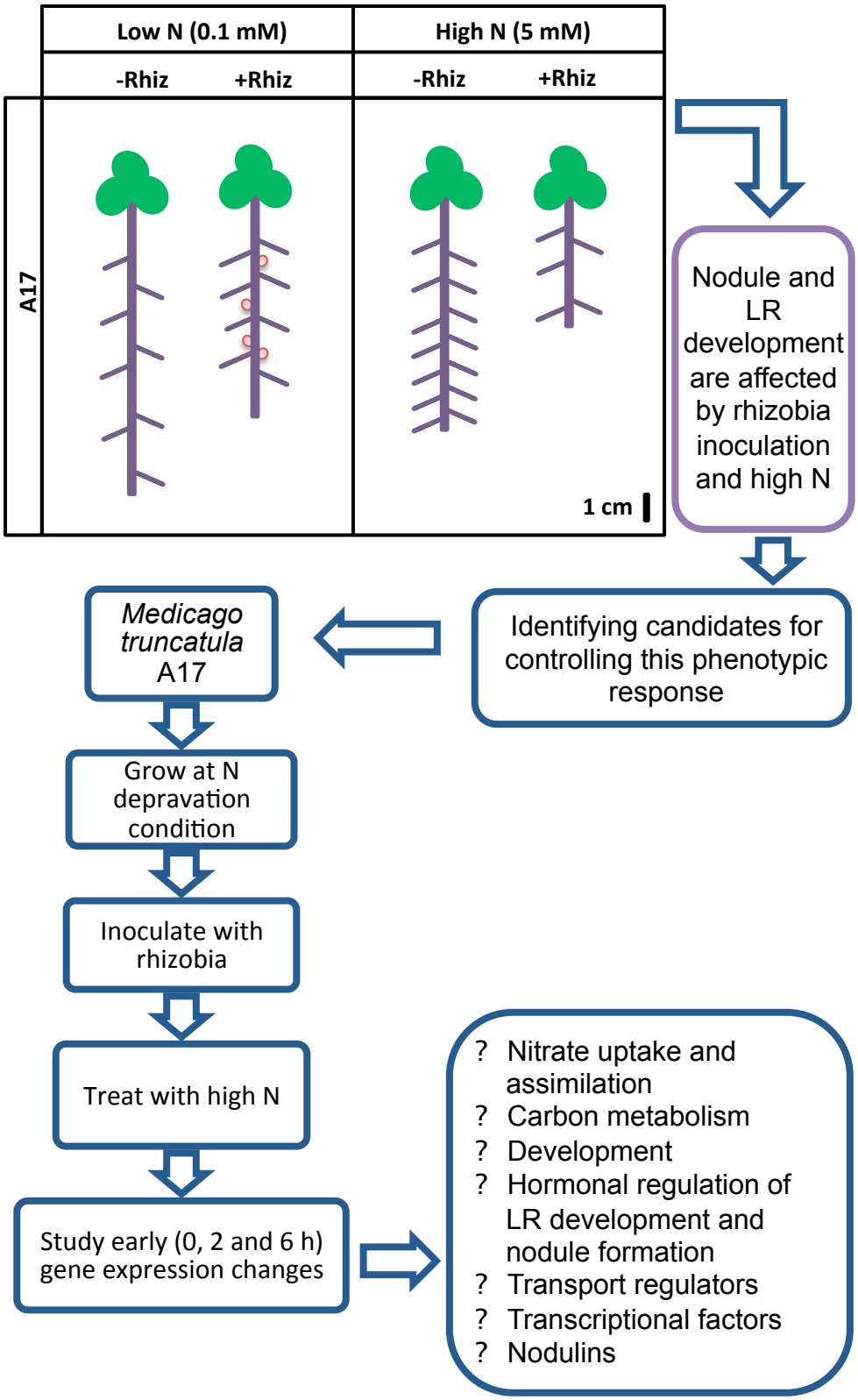
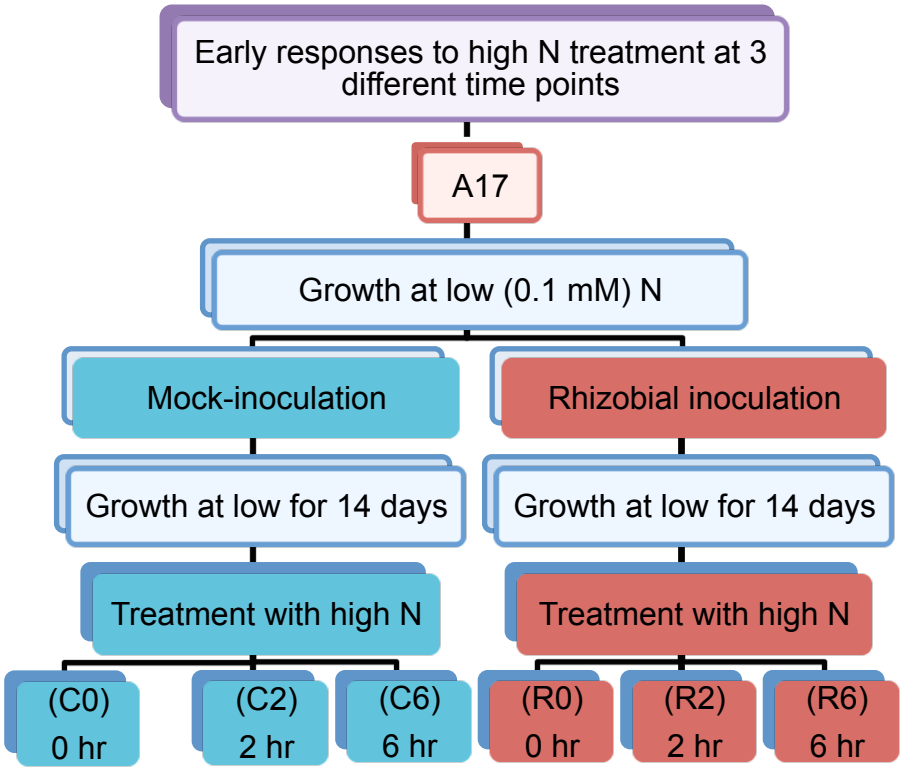


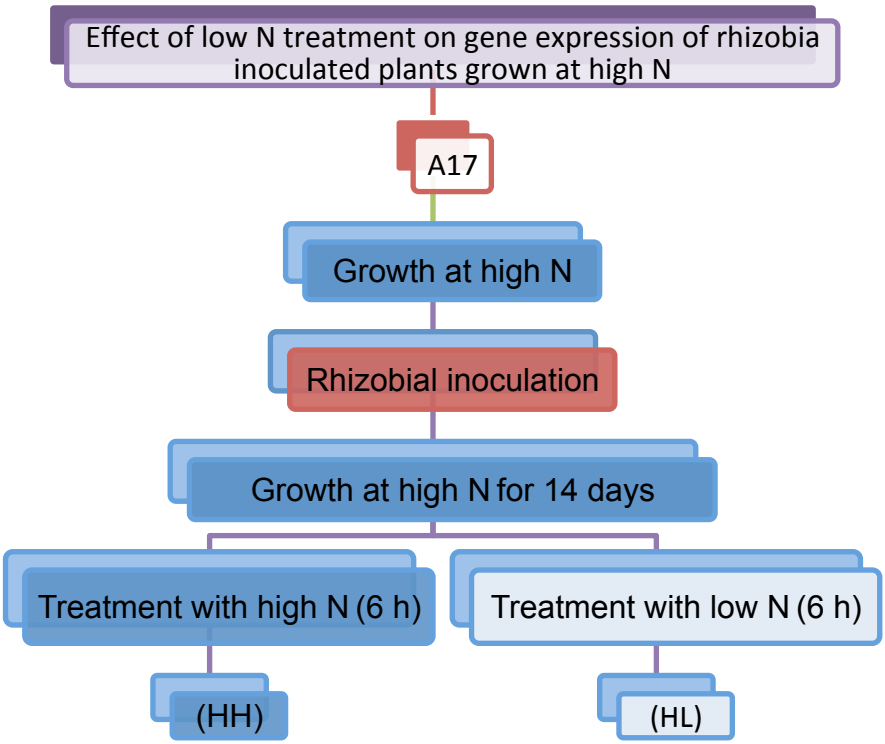
Figure 4-1: Schematic representation of effect of rhizobia and N on root architecture in A17 at low (0.1 mM) and high (5 mM) N; n=12 plants. Low N had no significant effect on PR and LR development in the presence or absence of rhizobia. The effect of high N on LR was significant in the presence of rhizobia. High N inhibited nodulation but rhizobia presence affected root development resulting in smaller root size with shorter PR and less total LR length that was due to lower number of LRs (the average of LR length was not significantly difference between treatments).

Figure 4-2

(a)


Early responses to high N treatment at 3 different time points
A17
Growth at low (0.1 mM) N
Mock-inoculation
Rhizobial inoculation
Growth at low for 14 days
Growth at low for 14 days
Treatment with high N
Treatment with high N
(C0) 0 hr
(C2) 2 hr
(C6) 6 hr
(R0) 0 hr
(R2) 2 hr
(R6) 6 hr

(b)


Effect of low N treatment on gene expression of rhizobia inoculated plants grown at high N
A17
Growth at high N
Rhizobial inoculation
Growth at high N for 14 days
Treatment with high N (6 h)
Treatment with low N (6 h)
(HH)
(HL)

(c)

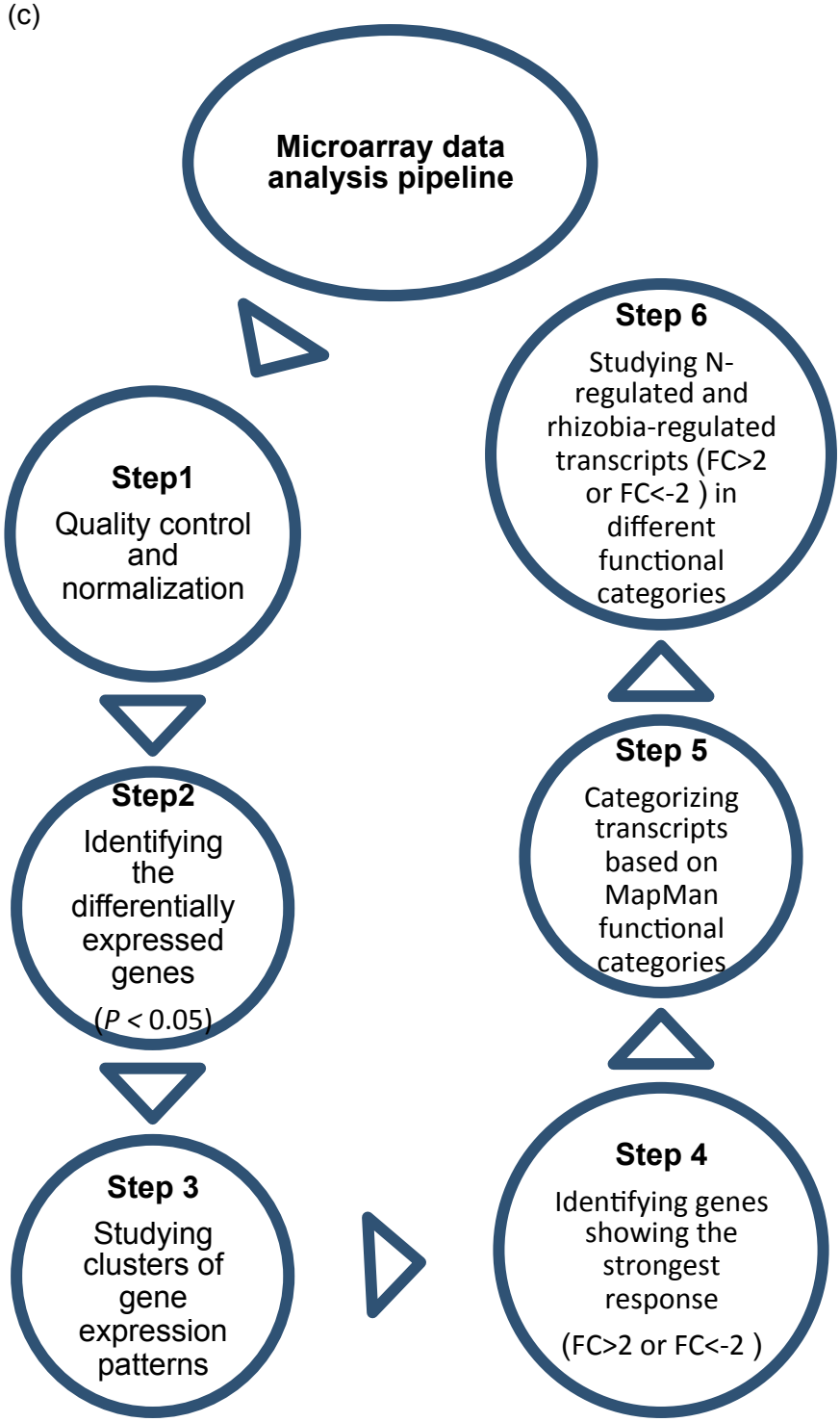


Figure 4-2: Experimental design for studying whole genome expression changes: a) 0, 2 and 6 hours after treatment with high N (5 mM) in rhizobia inoculated and mock inoculated plants grown at low N (0.1 mM); b) 6 hours post treatment with low N in rhizobia inoculated plants grown on high N; c) Steps for analysing the microarray data and identifying significantly affected genes by the treatments.

for each biological replicate (n=9 plants). The labeled cDNA samples were assigned to the microarrays on random (using randomizer tool at randomizer.org) to avoid technical bias.

To visualize the distribution of the data, i.e. XYS expression values that represented the coordinates of the probes on the microarrays (XY) and their intensity (S for signal), hist method and boxplot method were applied (Figure 4-3) using the R functions '`boxplot`' and '`hist`' (Carvalho and Irizarry, 2010). Both methods use the $\log_2$ values of the intensities and produce boxplots (Figure 4-3 a and c) or smoothed histograms (Figure 4-3 b and d) for each sample. To remove systematic variation from the expression data and render the measurements from different arrays inter-comparable, the robust multiarray average (RMA) algorithm was applied to the raw expression data (rma method in R). This algorithm performed background subtraction, quantile normalization and summarization via median polish (Carvalho, 2010).

To confirm replicate reproducibility distribution of the normalised data was visualized by plotting box plots (Figure 4-4 a and d) and smooth histograms (Figure 4-4 b and e) then comparisons were performed between replicates and across the data set. The overall microarray expression levels of the RMA normalised data were compared against each other by calculating the Pearson's *r* correlation coefficients for each pairwise comparison using the R function `cor`. The correlations were plotted on heatmaps (Figure 4-4 c and f) using R function 'heatmap.2'.

The pair-wise comparisons of the microarrays for the early time points after treatment of -Rhiz and +Rhiz seedlings grown at low N with high N (Figure 4-2 a) showed that there was a strong correlation between microarray replicate sets indicated by predominant green coloration in the heatmap (Figure 4-4 c) for a row or column with correlation ranging between $0.75 \leq r \leq 0.99$ with average $r = 0.94$. Replicate 2 of +Rhiz samples treated with high N for 6 hr (R6-2) had the lowest correlation with the other replicates with an average correlation of $r = 0.80$ (Figure 4-4 c). Replicate 3 of +Rhiz samples

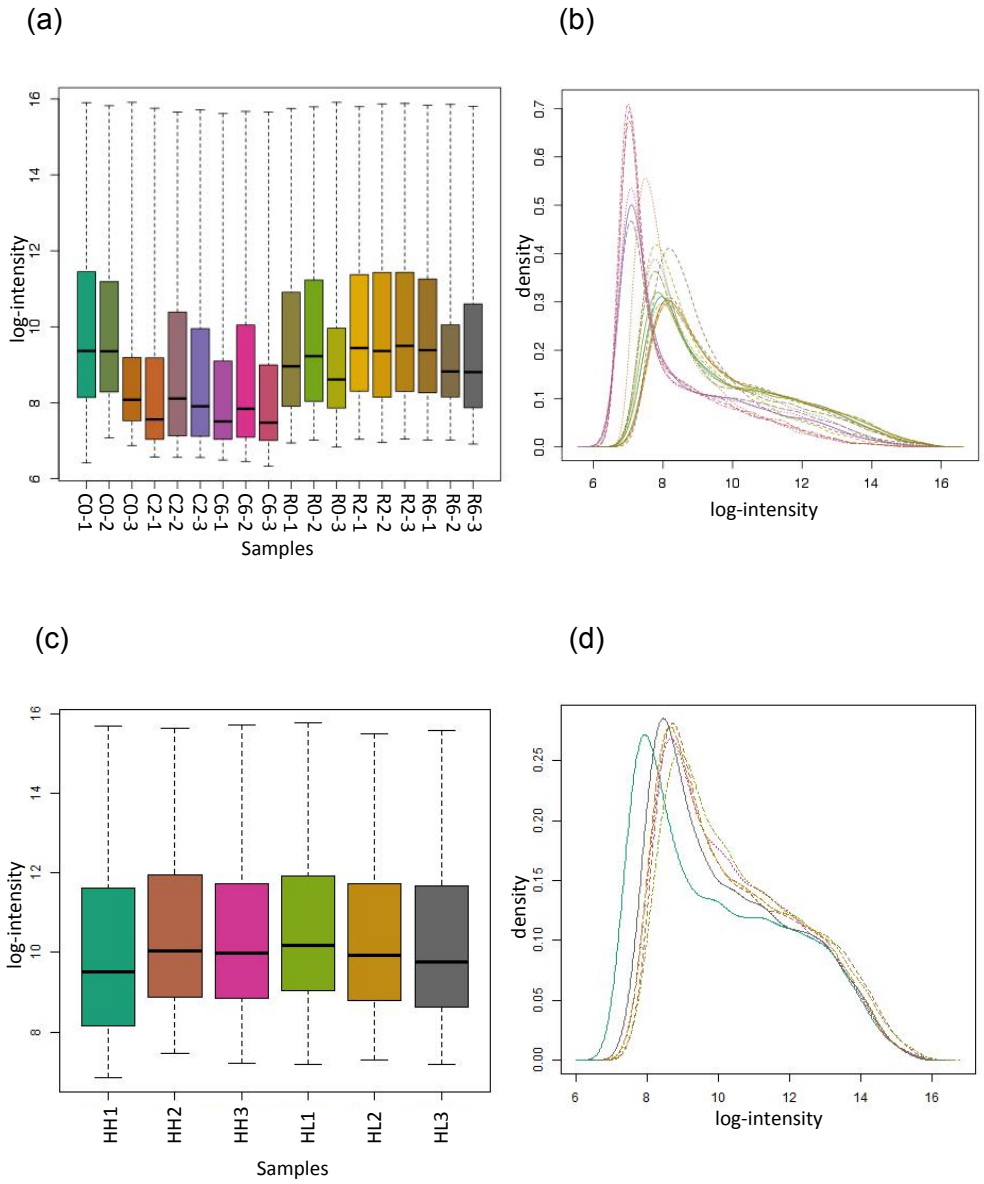
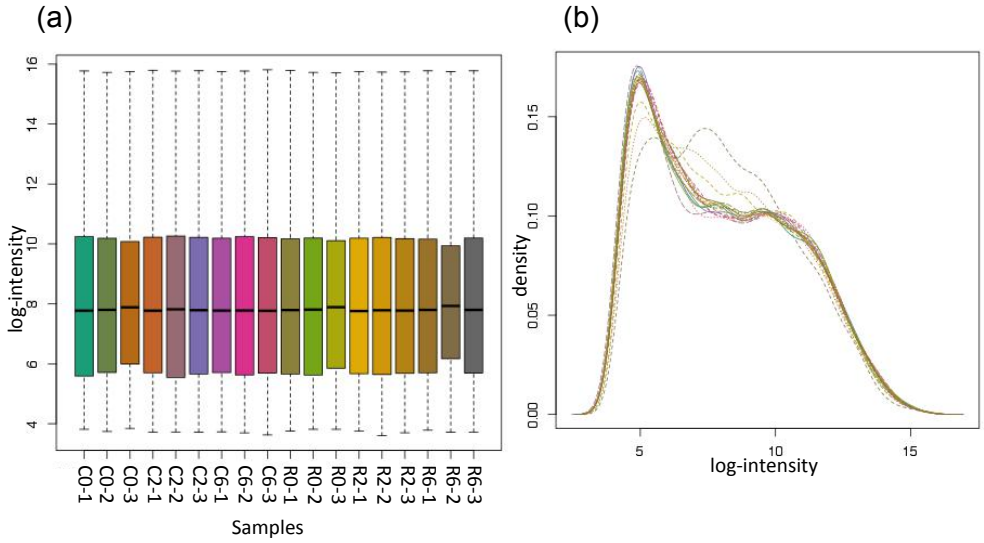
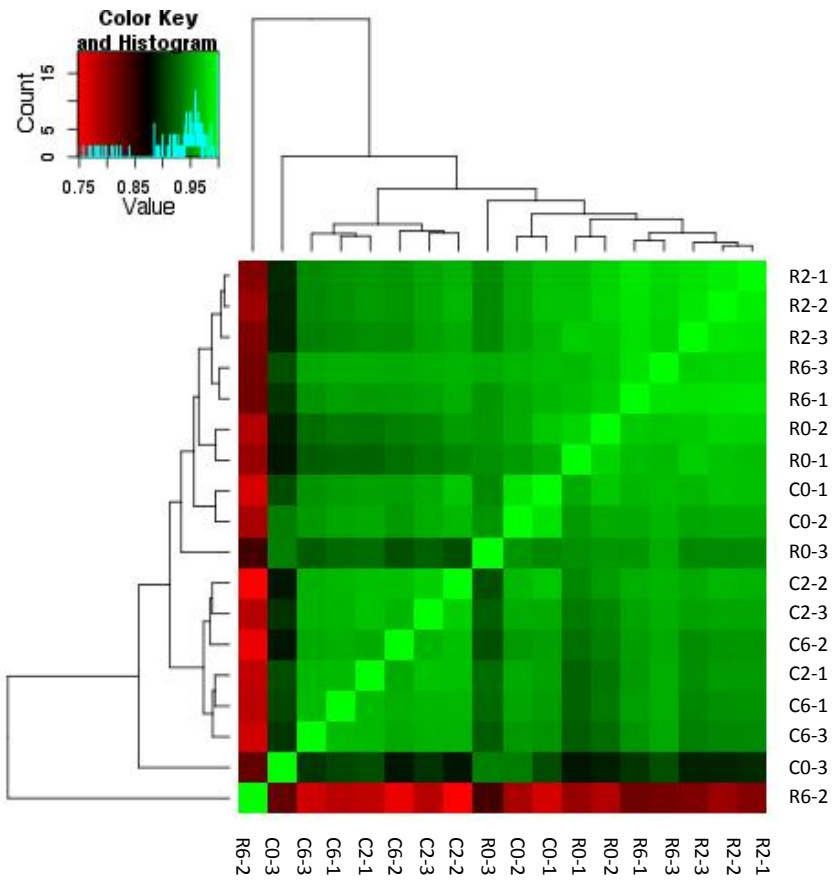


Figure 4-3: Distribution of log_2-intensities (XYS expression values) of 3 biological replicates of a-b) early time points (2 and 6 h) after high N treatment of rhizobia and mock inoculated plants grown at low N and c-d) low N treatment of rhizobia inoculated plants grown at high N, plotted as box plots (a and c) and smooth histograms (b and d). Labels correlate to the experimental design in figure 4-2.

Figure 4-4


(a)
log-intensity
Samples
C0-1
C0-2
C0-3
C2-1
C2-2
C2-3
C6-1
C6-2
C6-3
R0-1
R0-2
R0-3
R2-1
R2-2
R2-3
R6-1
R6-2
R6-3
(b)
density
log-intensity

(c)


Color Key and Histogram
Count
Value
R2-1
R2-2
R2-3
R6-3
R6-1
R0-2
R0-1
C0-1
C0-2
R0-3
C2-2
C2-3
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C6-1
C6-3
C0-3
R6-2

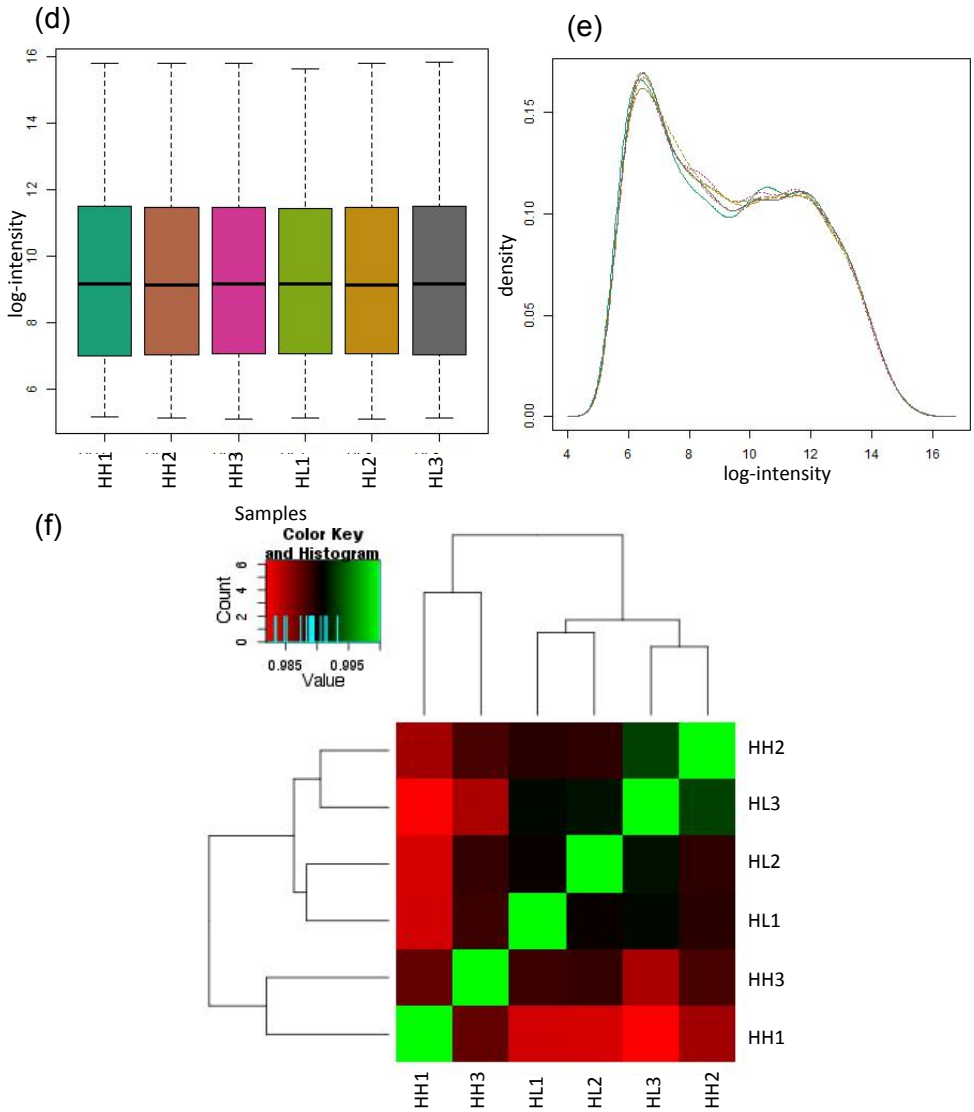


Figure 4-4: Distribution of log_2-intensities of normalized microarray data of a) early time points (2 and 6 h) post high N treatment of rhizobia and mock inoculated plants grown at low N and b) low N treatment of rhizobia inoculated seedlings grown at high N. Labels correlate to the experimental design in figure 4-2. The box plots (a and d) and histograms (b and e) gives an over view of the distribution of the data after normalization, showing all of the microarrays. c) Heatmap showing the level of correlation between all replicates of 0, 2 and 6 hr treatments with high N in rhizobia (R) or mock inoculated (C) except for R6-2 (anomalous replicate and removed post-normalisation). f) Heatmap showing the level of correlation between all replicates of rhizobia inoculated samples treated with low (HL) or high (HH) N. The heatmaps are symmetric about the diagonal, and reading by column or row is equivalent. The diagonal shows correlation between each sample and itself (= 1).

treated with high N for 6 hr (R6-3) showed the highest correlation with the other arrays with an average correlation of r = 0.96. Hence, R6-2 array was discarded from the analysis due to poor correlation with the other replicates and arrays, an indication of a poor quality array, most likely due to hybridization of that one array, since the RNA from that sample was found to be of good quality, and other biological sample arrays taken at the same time point had strong reproducibility. The poor quality of R6-2 was also evident in the normalised data plotted as boxplots and histogram in Figure 4-4 c.

The visualization of the inter-array correlation (Figure 4-4 f) for the second microarray experiment that looked at the effect of low N treatment on +Rhiz plants grown at high N (Figure 4-2 b) showed that expression comparisons of the replicates and arrays were all strong with $0.98 \leq r \leq 0.99$ and an average of r = 0.99. This correlation heatmap and the normalised expression values plotted as boxplots and histogram all confirm the good quality of the data.

4.2 Early responses to high N treatment in plants grown under low N in the presence or absence of rhizobia

This microarray experiment was designed to study early events and gene expression changes after treatment of plants grown under N limitation (low N, 0.1 mM) with high N (5 mM) in -Rhiz and +Rhiz (Figure 4-2 a). Gene expression changes were studied at 0, 2 and 6 hours after treatment with high N to identify genes involved in mediating the early N responses in root and balancing nodule and LR development in high N conditions. The hypothesis was that genes involved in nitrate uptake and assimilation such as nitrate and ammonium transporter genes, cell wall genes involved in stimulation and suppression of nodule formation in the cortex, cytokinin biosynthetic genes and transport regulators, auxin transporters and response factors and transcriptional factors would be candidates for controlling the phenotypic responses (Figure 4-1). The 0, 2, and 6 hours time points were chosen according to the previous published data on the root N (Gifford et al., 2008)

and nodulation (Lohar et al., 2006) response. Previous work has shown that a significant amount of N is acquired 2-4 h pt with similar levels of high nitrate (Cabeza et al., 2014), thus any transcriptomic changes triggered by N would reflect plant responses to N at these time points.

4.2.1 Genes rapidly respond to N treatment in both nodulating and non-nodulating *Medicago* plants

Hierarchical models are suitable models for data with small number of replicates by allowing sharing information between units of information (genes). In this study the hierarchical GaGa algorithm (Rossell, 2009) was applied to identify differentially expressed genes. First the groups each sample belonged to were defined: Control/-Rhiz (C) C0, C2, C6, Rhizobium-treated (R) R0, R2, R6, each in 3 replicates except for R6 in 2 replicates (as discussed in section 4.1); 0, 2, and 6 indicates hours after treatment with high N. Gene expression patterns (i.e. the desired hypothesis) were then generated for all combinations of patterns. For example, for three groups (or sample types) the patterns for gene expression (hypothesis) would be as follows, considering expression level in each group:

Pattern 0 (null hypotheses): group 1 = group 2 = group 3

Pattern 1 (alternative hypothesis): group 1 ≠ group 2 = group 3

Pattern 2: group 1 = group 2 ≠ group 3

Pattern 3: group 1 ≠ group 2 ≠ group 3

All possible patterns of comparisons between treatments were generated (203 patterns) and the data was then fitted to a hierarchical Gamma-Gamma model. The posterior probability (similar to FDR) that a gene followed each expression pattern was then derived to obtain lists of differentially expressed genes.

Elevated expression levels of genes involved in N acquisition and assimilation and root architecture changes (development of LRs and nodule

formation) at early time points after treatment with high N in the seedlings that were grown under low N conditions suggests an initiation of local N responses to take up N following previous N deprivation. A total of 4793 genes were identified as differentially expressed with a significance cutoff of $P<0.05$. This represented 10% of the genes on the Nimblegen Mt3.5 custom expression array and 7.4% of the genes annotated in the *M. truncatula* 3.5 gene model. This parallels comparable transcriptomic studies on *Arabidopsis* showing that the expression of up to 10% of the genome is under the control of nitrate and N supply (Bouguyon et al., 2012).

Around 3% of the differentially expressed genes were TFs some involved in stress responses, N responses, and LR and nodule development. Around 40% of the identified genes could be directly related to N responses (such as N assimilation and transport) or changes in root architecture (LR development and nodulation) and hormonal control of LR development and nodule formation.

4.2.2 An overview of the gene expression patterns showing N response and rhizobia effect

To study the expression patterns of the genes under the above conditions, differential expressed genes were clustered into 57 clusters (Figure 4-5). Clustering analysis revealed a range of different patterns of expression in response to this short-term high N treatment. The average expression level within each cluster that is plotted as a heatmap in Figure 4-5 a shows the distinct clustering between -Rhiz and +Rhiz samples. The clustering also suggests that both +Rhiz and -Rhiz samples treated with high N at 2 and 6 h are showing a more similar response than the 0 h which was immediately after the N deprivation period (branches i and ii in figure 4-5 a). An overall view of the heatmap suggests that N response was stronger than rhizobia response and most clusters are responding to N treatment independent of presence or absence of rhizobia.

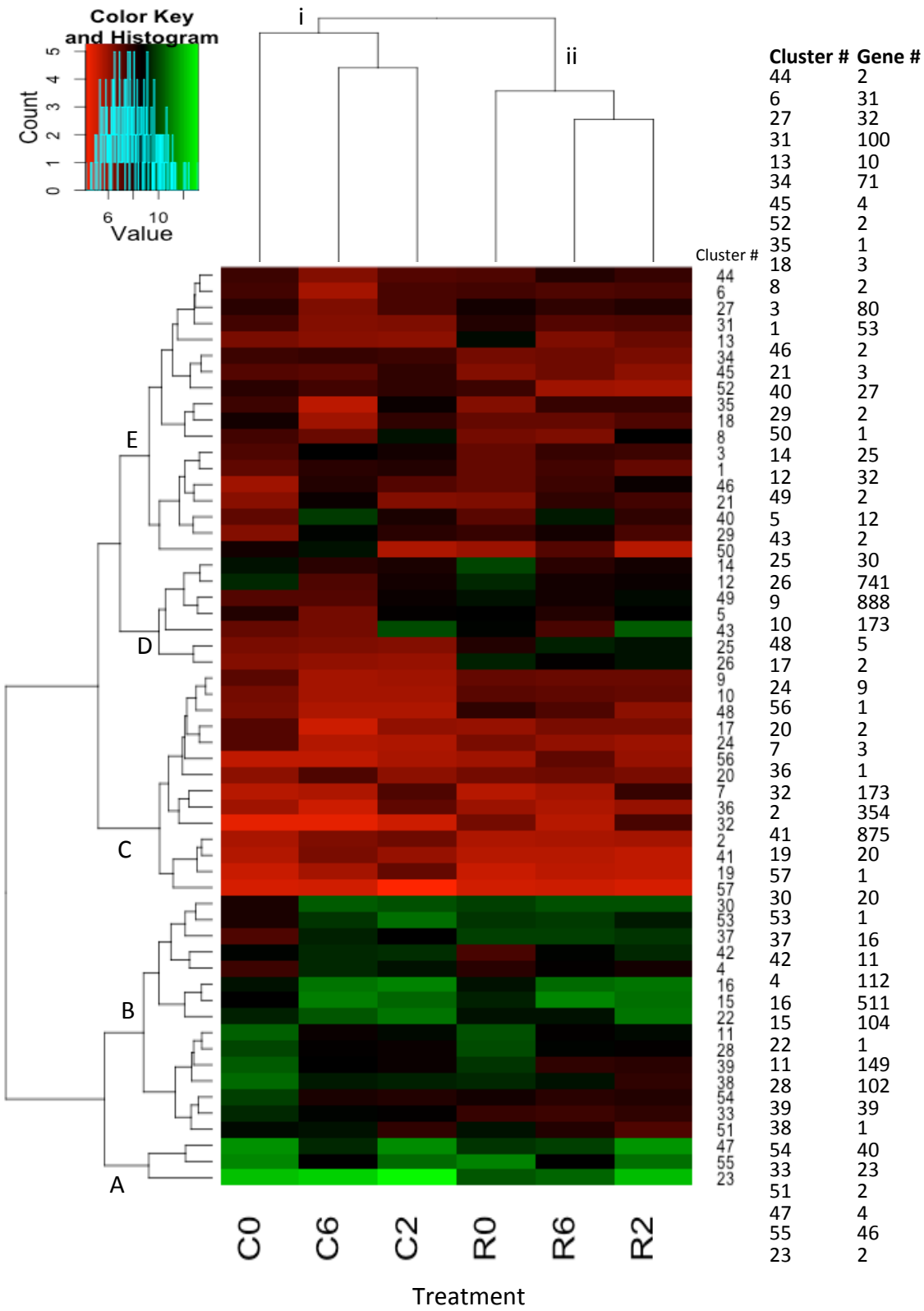
As plotted in the heatmap, 5 main responses to rhizobia inoculation and N treatment (branches A-E in Figure 4-5 a) could be identified. Clusters in branches A, B and C show similar response to N treatment both in +Rhiz and -Rhiz, indicating that the response is independent of the rhizobia effect. In clusters of branches B and C the gene expression level is different in +Rhiz and -Rhiz pt with high N. For example in cluster 11 (Figure 4-5 b) (149 genes) from branch B, -Rhiz and +Rhiz show similar response to N and gene expression level drops pt with high N both at 2 and 6-hour time points. Response to N could be suggested in this cluster as changes in expression levels of genes involved in N assimilation (such as amino acid permease and glutamate receptor). In addition the cluster includes genes involved in the biosynthesis of auxin, cytokinin and gibberellin and several members of the bHLH TF family, suggesting that N regulates hormone signaling, hormone metabolism and developmental processes. In branch C cluster 2 (Figure 4-5 b) with 354 genes shows that early after treatment with high N, gene expression level changes in both -Rhiz and +Rhiz samples – this is therefore a Rhizobium-independent N response that is maintained at 2 and 6 hr, however the average of gene expression level in this cluster is higher in -Rhiz compared to +Rhiz. The presence of genes involved in LR development such as Medtr2g038450.1 Auxin efflux carrier component, Medtr4g076670.1 Cell division protein kinase, Medtr4g075380.1 Ethylene-responsive TF and Medtr5g080470.1 LOB domain-containing protein may suggest root architecture changes in response to N. Clusters in branch D mainly show rhizobia effects in response to N treatment and in branch E expression levels are different between +Rhiz and -Rhiz in response to N treatment.

4.2.3 Identifying genes showing the strongest response to N treatment and rhizobia inoculation

To study the responses plotted in Figure 4-5 a in more detail and to identify the genes that showed strong responses to high N treatment at 2 and 6 h or/and were affected by rhizobia at low N (0 h) or high N treatment (2 and 6 h), FC of the expression values of the differentially expressed genes were

Figure 4-5

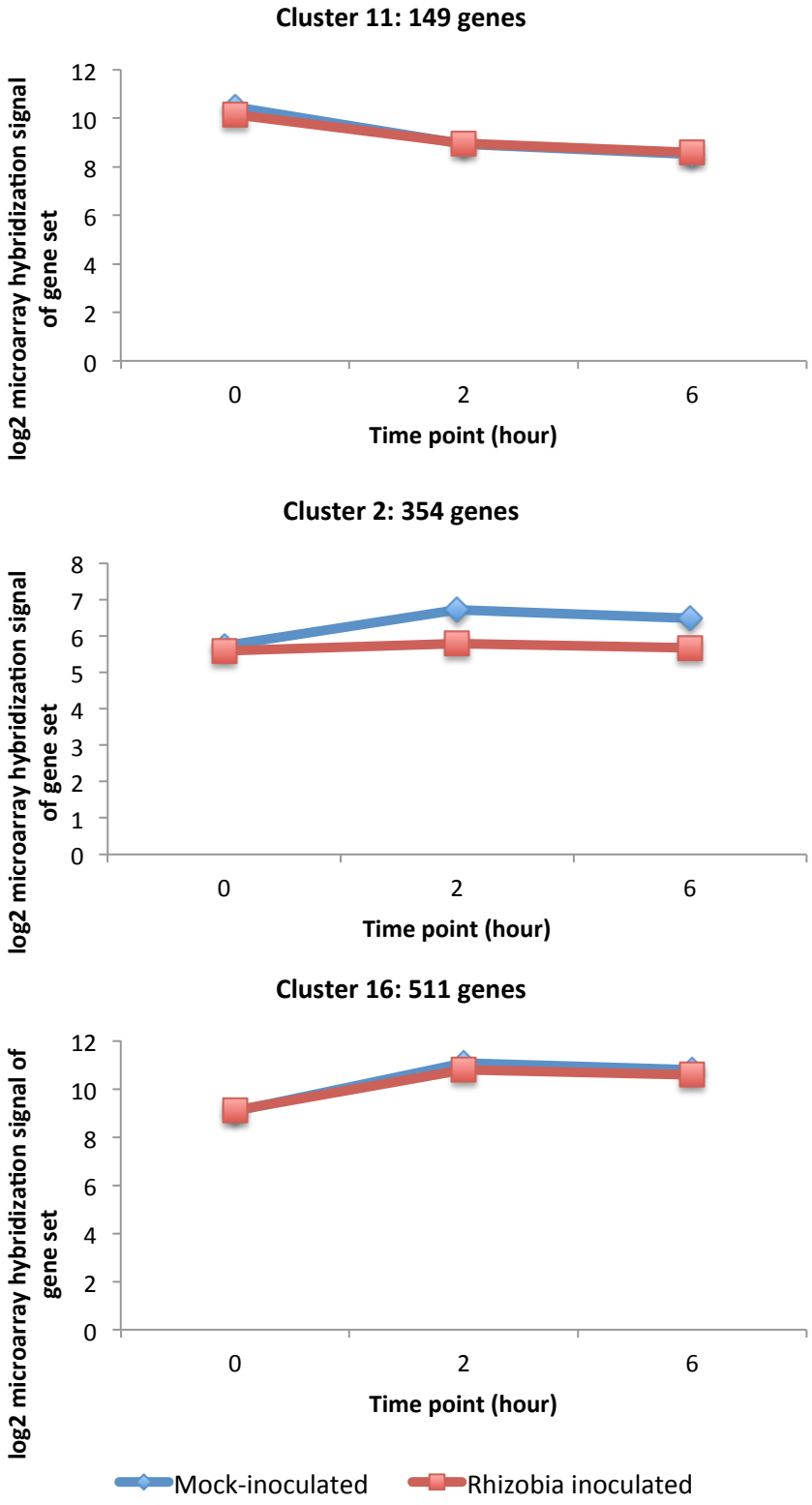
(a)



Cluster #	Gene #
44	2
6	31
27	32
31	100
13	10
34	71
45	4
52	2
35	1
18	3
8	2
3	80
1	53
46	2
21	3
40	27
29	2
50	1
14	25
12	32
49	2
5	12
43	2
25	30
26	741
9	888
10	173
48	5
17	2
24	9
56	1
20	2
7	3
36	1
32	173
2	354
41	875
19	20
57	1
30	20
53	1
37	16
42	11
4	112
16	511
15	104
22	1
11	149
28	102
39	39
38	1
54	40
33	23
51	2
47	4
55	46
23	2

Figure 4-5, continue

(b)



(b), continue

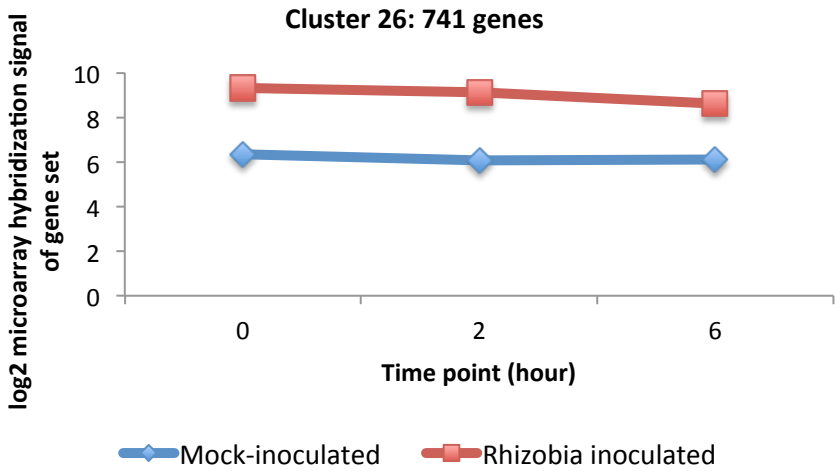


Figure 4-5: Expression patterns of clusters of genes differentially expressed in response to high N (5 mM) treatment and rhizobial inoculation at 0, 2 and 6-hour time points plotted as the average of $\log_2$ microarray hybridization signal of genes for each cluster. a) Heatmap showing expression patterns of all gene clusters (rows) at different high N treatment time points (columns); b) average of gene expression values in clusters 11, 2, 16 and 26 in -Rhiz and +Rhiz samples in response to high N treatment.

calculated. This was done specifically to identify (1) Early responses to N in -Rhiz and +Rhiz samples 2 and 6 hours pt with high N; (2) The effect of rhizobia on the samples treated with high N for 2 and 6 hours; (3) The effect of rhizobia at 14 dpi on samples grown at low N.

To study early responses to high N treatment, fold changes (FC) between C0 and C2, C0 and C6, C2 and C6, R0 and R2, R0 and R6, R2 and R6 were calculated. This enabled quantitative understanding of gene expression changes at 2, and 6 hr and between 2 to 6 hours after treatment with high N in -Rhiz and +Rhiz respectively (Tables 4-1 and 4-2 a). FC between C2 and R2 and also C6 and R6 showed the effect of rhizobia on gene expression in samples treated with high N after 2 and 6 hours respectively (Tables 4-1 and 4-2 b). FC between C0 and R0 showed changes in gene expression 14 days post inoculation with rhizobia, compared to plants grown at low N without rhizobia (Tables 4-1 and 4-2 b).

In the next step patterns of differentially expressed genes in different biological pathways were analysed using the MapMan software to identify specific metabolic and molecular processes affected by rhizobia and/or N (Thimm et al., 2004). MapMan software uses the Wilcoxon Rank Sum Test to predict functional categories (BINs) that show a different behaviour in terms of expression profile compared to all the other remaining BINs. To study early N responses, pathway analysis of all the major pathways (BINs) was performed by applying the Wilcoxon rank sum test with Benjamini-Hochberg correction (Thimm et al., 2004). Significantly different (P<0.05) functional pathways (bins and sub-bins) containing N-regulated (Table 4-3 a) and rhizobia regulated (Table 4-3 b) genes were selected and would be discussed below.

The gene expression patterns of the amino acid metabolism, N metabolism and stress pathways show that -Rhiz samples had a slightly stronger response to high N compared to +Rhiz (Figure 4-6). Genes involved in N metabolism pathway were upregulated both in -Rhiz and +Rhiz samples after 2 and 6 hours pt. Genes involved in amino acid metabolism also showed

Table 4-1: Number of genes showing significant (FC>2 or FC<-2) N response at 2, 6 and between 2-6 hours post treatment with high N in mock inoculated and rhizobia inoculated samples and number of genes affected by rhizobia (FC>2 or FC<-2) immediately (0 hr), 2 and 6 hours after treatment with high N.

Response	**Number of significantly expressed genes (FC>2 or FC<-2)**					
N response	**Mock-inoculated**			**Rhizobia inoculated**		
	T	**U**	**D**	**T**	**U**	**D**
High N effect at 2 h	440	307	133	229	171	58
High N effect at 6 h	570	314	256	298	154	144
High N effect between 2-6 h	57	18	39	50	39	11
Rhizobia effect	**T**		**U**		**D**	
Rhizobia effect at 0 h	553		534		19	
Rhizobia effect at 2 h	516		494		22	
Rhizobia effect at 6 h	484		441		43	

T= Total, U= Upregulated, D= Downregulated

Table 4-2: Significantly expressed (FC>2 or FC<-2) genes showing different or similar responses to a) high N and/or b) rhizobia at 0, 2 and 6-hour time points. Different shades of blue: upregulated genes and different shades of yellow: downregulated genes.

(a)

N response				
2 hr	6 hr	2-6 hr	Number of genes	
			52	Mock inoculated
			13	
			26	
			112	
			40	
			52	
			1	
			2	
			3	
			5	
			9	
			16	
			2	
			1	
			2	
			10	
			1	
			2	
			1	
			1	
			3	
			2	
			23	Rhizobia inoculated
			3	
			17	
			56	
			30	
			17	
			1	
			1	
			1	
			1	
			6	
			6	
			15	
			1	
			1	
			9	
			1	
			2	
			1	

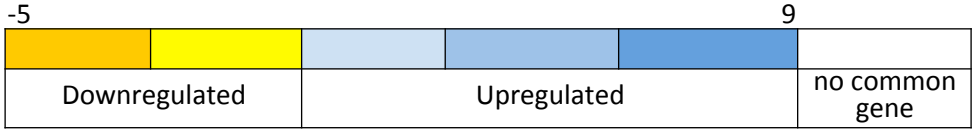


Table 4-2, (b)

Rhizobia effect			
0 hr	2 hr	6 hr	Number of genes
			163
			75
			175
			2
			1
			138
			142
			66
			2
			1
			1
			117
			213
			36
			45
			69
			17
			4
			34
			12
			75
			16
			4
			3
			12
			30
			2

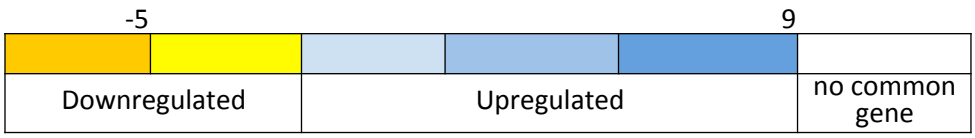


Table 4-3: N-regulated (a) and rhizobia-regulated (b) biological categories as analysed by Mapman (Wilcoxon rank sum test with Benjamini-Hochberg corrected; $P < 0.05$). Elements= number of genes in each category, -Rhiz= mock inoculated and +Rhiz= rhizobia-inoculated samples at 0, 2 or 6 hour time points.

(a)

	N-regulated			
	Bin	Name	Elements	p-value
-Rhiz 2 h	3.5	minor CHO metabolism.others	4	0.0546
	8	TCA / org. transformation	14	0.0057
	8.3	TCA / org. transformation.carbonic anhydrases	11	0.0484
	12	N-metabolism	9	0.0029
	12.1	N-metabolism.nitrate metabolism	5	0.0308
	13	amino acid metabolism	42	2.7147 E-05
	13.1	amino acid metabolism.synthesis	22	0.0320
	13.1.1	amino acid metabolism.synthesis.central amino acid metabolism	7	0.0432
	13.1.1.3	amino acid metabolism.synthesis.central amino acid metabolism.alanine	7	0.0432
	13.1.1.3.11	amino acid metabolism.synthesis.central amino acid metabolism.alanine.alanine-glyoxylate aminotransferase	5	0.0201
	13.2	amino acid metabolism.degradation	19	0.0002
	13.2.4	amino acid metabolism.degradation.branched chain group	9	0.0003
	13.2.4.4	amino acid metabolism.degradation.branched-chain group.leucine	7	0.0020
	16	secondary metabolism	91	4.0708 E-05
	16.1	secondary metabolism.isoprenoids	31	2.0577 E-05
	16.1.2	secondary metabolism.isoprenoids.mevalonate pathway	11	0.0204
	16.1.2.3	secondary metabolism.isoprenoids.mevalonate pathway.HMG-CoA reductase	7	0.0005
	16.1.5	secondary metabolism.isoprenoids.terpenoids	15	0.0057
	16.8	secondary metabolism.flavonoids	18	0.0634
	16.8.1	secondary metabolism.flavonoids.anthocyanins	6	0.0555
	17.5	hormone metabolism.ethylene	17	0.0241
	20	stress	143	0.0015
	20.1	stress.biotic	96	0.0204
	20.1.7	stress.biotic.PR-proteins	50	0.0580
	21.1	redox.thioredoxin	7	0.0555
	21.3	redox.heme	12	0.0320
	26.1	misc.misc2	12	0.0449
	26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	6	0.0201

Table 4-3 a, continue

	N-regulated			
	Bin	Name	Elements	p-value
-Rhiz 2 h	26.12	misc.peroxidases	20	0.0204
	27	RNA	234	0.0002
	27.3	RNA.regulation of transcription	213	0.0007
	27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	6	0.0065
	27.3.35	RNA.regulation of transcription.bZIP transcription factor family	10	0.0045
	27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	8	0.0046
	28.2	DNA.repair	6	0.0204
	34.4	transport.nitrate	4	0.0269
	35	not assigned	2944	4.9854 E-11
	35.2	not assigned.unknown	2944	4.9854 E-11
-Rhiz 6 h	1.3	PS.calvin cycle	14	0.0475
	1.3.2	PS.calvin cycle.rubisco small subunit	7	0.0060
	5	fermentation	6	0.0426
	8	TCA / org. transformation	14	0.0511
	12	N-metabolism	9	0.0046
	12.1	N-metabolism.nitrate metabolism	5	0.0318
	13	amino acid metabolism	42	0.0002
	13.1	amino acid metabolism.synthesis	22	0.0265
	13.1.1.3.11	amino acid metabolism.synthesis.central amino acid metabolism.alanine.alanine-glyoxylate aminotransferase	5	0.0438
	13.2	amino acid metabolism.degradation	19	0.0031
	13.2.4	amino acid metabolism.degradation.branched chain group	9	0.0009
	13.2.4.4	amino acid metabolism.degradation.branched-chain group.leucine	7	0.0066
	16	secondary metabolism	91	5.4268 E-06
	16.1	secondary metabolism.isoprenoids	31	0.0001
	16.1.2	secondary metabolism.isoprenoids.mevalonate pathway	11	0.0511
	16.1.2.3	secondary metabolism.isoprenoids.mevalonate pathway.HMG-CoA reductase	7	0.0070
	16.1.5	secondary metabolism.isoprenoids.terpenoids	15	0.0104
	16.2.1.9	secondary metabolism.phenylpropanoids.lignin biosynthesis.COMT	5	0.0438
	16.8	secondary metabolism.flavonoids	18	0.0195
	16.8.1	secondary metabolism.flavonoids.anthocyanins	6	0.0066
	16.8.1.21	secondary metabolism.flavonoids.anthocyanins.anthocyanin 5-aromatic acyltransferase	5	0.0215

Table 4-3 a, continue

	N-regulated			
	Bin	Name	Elements	p-value
-Rhiz 6 h	17.7	hormone metabolism.jasmonate	12	0.0382
	20	stress	143	0.0801
	26.1	misc.misc2	12	0.0584
	26.13	misc.acid and other phosphatases	14	0.0031
	27	RNA	234	0.0003
	27.3	RNA.regulation of transcription	213	0.0007
	27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	6	0.0071
	27.3.35	RNA.regulation of transcription.bZIP transcription factor family	10	0.0318
	27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	8	0.0265
	28.2	DNA.repair	6	0.0363
	35	not assigned	2944	0.0007
	35.2	not assigned.unknown	2944	0.0007
+Rhiz 2 h	1	PS	34	0.0568
	1.2	PS.photorespiration	4	0.0499
	1.3	PS.calvin cycle	14	0.0215
	9.4	mitochondrial electron transport / ATP synthesis.alternative oxidase	4	0.0568
	10.6	cell wall.degradation	20	0.0549
	10.7	cell wall.modification	5	0.0499
	12	N-metabolism	9	0.0016
	12.1	N-metabolism.nitrate metabolism	5	0.0216
	12.1.1	N-metabolism.nitrate metabolism.NR	4	0.0509
	13	amino acid metabolism	42	0.0016
	13.1.1	amino acid metabolism.synthesis.central amino acid metabolism	7	0.0568
	13.1.1.3	amino acid metabolism.synthesis.central amino acid metabolism.alanine	7	0.0568
	13.1.1.3.11	amino acid metabolism.synthesis.central amino acid metabolism.alanine.alanine-glyoxylate aminotransferase	5	0.0230
	13.2	amino acid metabolism.degradation	19	0.0037
	13.2.4	amino acid metabolism.degradation.branched chain group	9	0.0016
	13.2.4.4	amino acid metabolism.degradation.branched-chain group.leucine	7	0.0058
	16	secondary metabolism	91	0.0016
	16.1	secondary metabolism.isoprenoids	31	0.0007
	16.1.2	secondary metabolism.isoprenoids.mevalonate pathway	11	0.0229
	16.1.2.3	secondary metabolism.isoprenoids.mevalonate pathway.HMG-CoA reductase	7	0.0037
	16.1.5	secondary metabolism.isoprenoids.terpenoids	15	0.0100
	26.1	misc.misc2	12	0.0499

Table 4-3 a, continue

	N-regulated			
	Bin	Name	Elements	p-value
+Rhiz 2 h	26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	6	0.0057
	26.12	misc.peroxidases	20	0.0585
	26.13	misc.acid and other phosphatases	14	0.0037
	26.4	misc.beta 1,3 glucan hydrolases	10	0.0499
	27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	6	0.0266
	27.3.35	RNA.regulation of transcription.bZIP transcription factor family	10	0.0229
	27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	8	0.0181
	29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	36	0.0499
	30.2.19	signalling.receptor kinases.legume-lectin	3	0.0597
	30.2.8	signalling.receptor kinases.leucine rich repeat VIII-1	5	0.0509
	34.12	transport.metal	12	0.0568
	34.19	transport.Major Intrinsic Proteins	7	0.0509
	34.4	transport.nitrate	4	0.0243
+Rhiz 6 h	1.2	PS.photorespiration	4	0.0423
	1.3	PS.calvin cycle	14	0.0484
	3.4.3	minor CHO metabolism.myo-inositol.InsP Synthases	4	0.0429
	9.4	mitochondrial electron transport / ATP synthesis.alternative oxidase	4	0.0538
	12	N-metabolism	9	0.0027
	12.1	N-metabolism.nitrate metabolism	5	0.0305
	13	amino acid metabolism	42	0.0004
	13.1	amino acid metabolism.synthesis	22	0.0360
	13.1.1	amino acid metabolism.synthesis.central amino acid metabolism	7	0.0590
	13.1.1.3	amino acid metabolism.synthesis.central amino acid metabolism.alanine	7	0.0590
	13.1.1.3.11	amino acid metabolism.synthesis.central amino acid metabolism.alanine.alanine-glyoxylate aminotransferase	5	0.0305
	13.2	amino acid metabolism.degradation	19	0.0043
	13.2.4	amino acid metabolism.degradation.branched chain group	9	0.0014
	13.2.4.4	amino acid metabolism.degradation.branched-chain group.leucine	7	0.0043
	16	secondary metabolism	91	0.0003
	16.1	secondary metabolism.isoprenoids	31	0.0004
	16.1.2	secondary metabolism.isoprenoids.mevalonate pathway	11	0.0360
	16.1.2.3	secondary metabolism.isoprenoids.mevalonate pathway.HMG-CoA reductase	7	0.0043
	16.1.5	secondary metabolism.isoprenoids.terpenoids	15	0.0113
	16.2.1.9	secondary metabolism.phenylpropanoids.lignin biosynthesis.COMT	5	0.0551

Table 4-3 a, continue

	N-regulated			
	Bin	Name	Elements	p-value
+Rhiz 6 h	17.7	hormone metabolism.jasmonate	12	0.0033
	17.7.1	hormone metabolism.jasmonate.synthesis-degradation	11	0.0074
	17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	10	0.0224
	21.3	redox.heme	12	0.0036
	26.1	misc.misc2	12	0.0423
	26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	6	0.0177
	26.12	misc.peroxidases	20	0.0356
	26.13	misc.acid and other phosphatases	14	0.0010
	26.4	misc.beta 1,3 glucan hydrolases	10	0.0581
	27	RNA	234	0.0360
	27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	6	0.0360
	29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	36	0.0360
	30.2.8	signalling.receptor kinases.leucine rich repeat VIII-1	5	0.0305
	30.5	signalling.G-proteins	11	0.0590
	34.4	transport.nitrate	4	0.0305
	34.19	transport.Major Intrinsic Proteins	7	0.0305
	34.19.3	transport.Major Intrinsic Proteins.NIP	6	0.0431

Table 4-3, (b)

	Rhizobia-regulated			
	Bin	Name	Elements	p-value
High N (2 h)	20	stress	143	4.7666E-06
	20.1	stress.biotic	96	0.0003
	20.1.7	stress.biotic.PR-proteins	50	0.0129
	21.3	redox.heme	12	3.6410E-06
	27	RNA	234	0.0006
	27.3	RNA.regulation of transcription	213	0.0023
	30	signalling	180	0.0129
	30.2	signalling.receptor kinases	115	0.0023
	30.2.24	signalling.receptor kinases.S-locus glycoprotein like	11	0.0603
	34	transport	155	0.0382
	34.13	transport.peptides and oligopeptides	18	0.0004
	35	not assigned	2944	3.6410E-06
	35.2	not assigned.unknown	2944	3.6410E-06

Table 4-3 b, continue

	Rhizobia-regulated			
	Bin	Name	Elements	p-value
High N (6 h)	20	stress	143	0.0026
	20.1	stress.biotic	96	0.0023
	21.3	redox.heme	12	1.3865E-05
	27	RNA	234	0.0024
	27.3	RNA.regulation of transcription	213	0.0042
	30.2	signalling.receptor kinases	115	0.0079
	34.13	transport.peptides and oligopeptides	18	0.0012
	35	not assigned	2944	0.0001
	35.2	not assigned.unknown	2944	0.0001
Low N (0 h)	8	TCA / org. transformation	14	0.0115
	8.3	TCA / org. transformation.carbonic anhydrases	11	0.0126
	16	secondary metabolism	91	0.0004
	16.1	secondary metabolism.isoprenoids	31	0.0002
	16.1.2.3	secondary metabolism.isoprenoids.mevalonate pathway.HMG-CoA reductase	7	0.0125
	16.1.5	secondary metabolism.isoprenoids.terpenoids	15	0.0027
	16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	14	0.0468
	17.2.1	hormone metabolism.auxin.synthesis-degradation	4	0.0434
	20	stress	143	0.0004
	20.1	stress.biotic	96	0.0001
	20.1.7	stress.biotic.PR-proteins	50	0.0031
	21	redox	33	0.0037
	21.3	redox.heme	12	1.9907E-05
	28.2	DNA.repair	6	0.0440
	30.2	signalling.receptor kinases	115	0.0126
	34	transport	155	0.0434
	34.13	transport.peptides and oligopeptides	18	0.0083

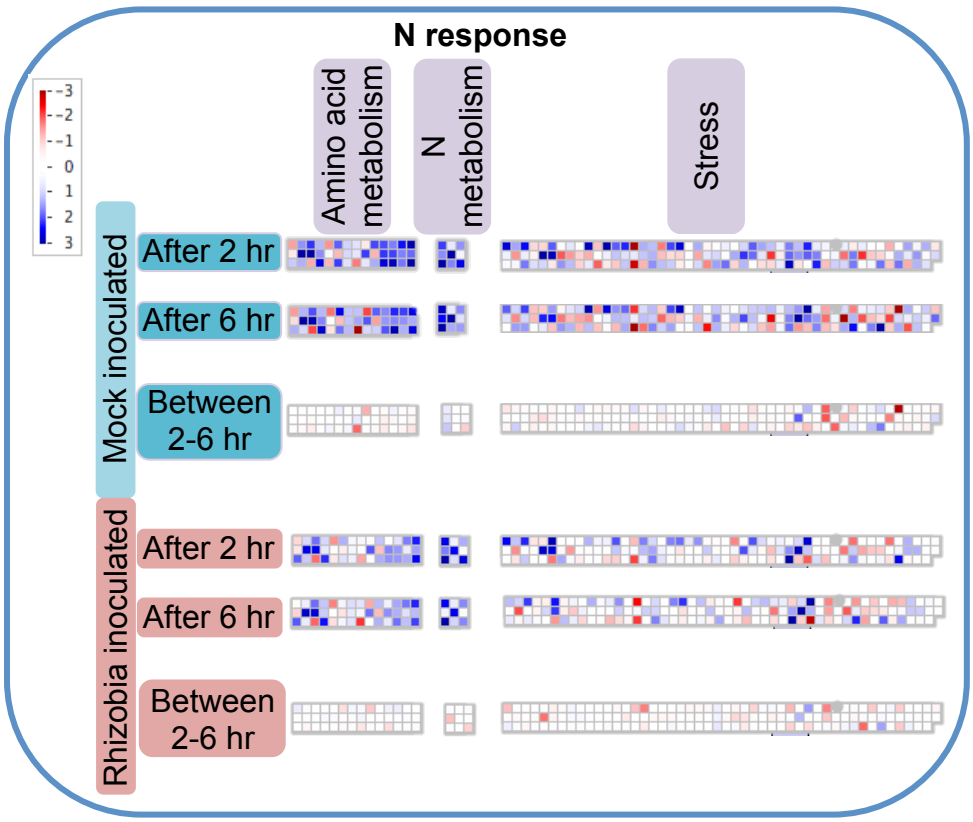


Figure 4-6: Gene expression responses to high N in three MapMan (Thimm et al., 2004) functional categories. The expression levels of upregulated (blue) and downregulated (red) genes are represented in BINs of gene function related to amino acid metabolism, N metabolism and stress pathways, determined using the International Medicago Genome Annotation Group (IMGAG) annotation. Mock inoculated plants had a slightly stronger response to high N treatment compared to rhizobia inoculated plant responses to N.

strong expression changes at these time points with the genes predominantly showing upregulation. Genes assigned to the stress category also showed strong response at 2 and 6-hour time points with the majority of genes being upregulated (Figure 4-6). These gene expression changes were not as strong between 2-6 hours.

To identify the genes that showed the strongest expression changes under the experimental conditions, differentially expressed genes with FC >2 or FC <-2 were selected as significant and considered for further analysis. Looking at the early responses to N (2, 6 and between 2-6 hours post pt) shows that in -Rhiz, 440 differentially expressed genes showed significant (FC>2 or FC<-2) response to high N at 2 h. This number of regulated genes was slightly higher (570 genes) at 6 h of which 55% of the genes were upregulated. Hence, the predominant effect was N-induction, particular at 2 h where 70% of genes were N-induced (Table 4-1). There was not a major gene expression change between 2 and 6 hours and only 57 differentially expressed genes showed strong response (FC>2 or FC<-2) to high N treatment with 68% of them being downregulated (Table 4-1). The number of differentially expressed genes showing strong N-regulated gene expression changes in +Rhiz was less than -Rhiz but showed almost the same trend in the number of genes being up or down regulated as -Rhiz (Table 4-1). Similar to -Rhiz, a higher percentage of genes were induced at 2 h (75%) compared to 6 h (52%). At 6-hour of N-treatment 298 differentially expressed gene showed strong response (FC>2 or FC<-2) to N treatment (52% upregulated). Between 2 and 6 hours 50 differentially expressed genes had strong (FC>2 or FC<-2) expression changes showing a strong downregulation (78%), (Table 4-1).

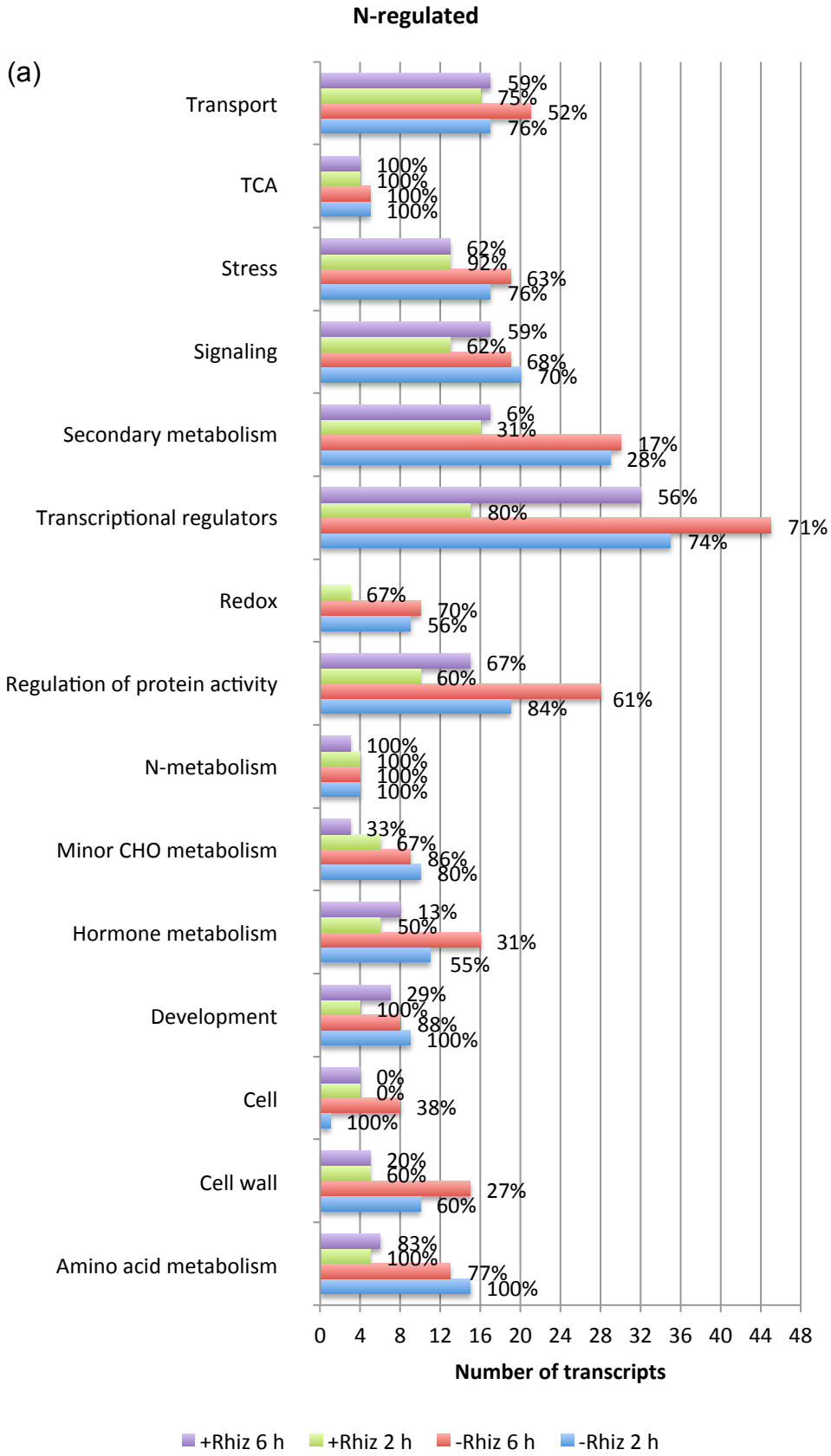
From these studies it could be concluded that high N effect was stronger in the absence of rhizobia (at both 2 and 6 h) with a higher number of genes showing N-regulated gene expression changes in -Rhiz than +Rhiz. The response trend was however similar in respect to the number of genes induced or repressed. N-regulation was stronger at 2 h than 6 h with a higher

percentage of significantly expressed genes being N-induced in -Rhiz and +Rhiz. Thus the studies suggest that the early (2 and 6 h) response to high N is mainly independent of the rhizobia effect and N-induction is the predominant effect with genes prominently induced at 2 h time point. In response to nitrate a rapid (within minutes) transcriptional response is induced which does not require protein synthesis and thus it is known as the Primary Nitrate Response (PNR) (Redinbaugh and Campbell, 1991, Krouk et al., 2010b). PNR affects a wide range of gene functional categories such as ion transport, primary and secondary metabolism, biosynthesis of nucleic acids, transcription and RNA processing, and hormone homeostasis (Krouk et al., 2010a, Krouk et al., 2010b). In this study some of the main gene functional categories ($P < 0.05$) responding to the N supply in the form of NH_4NO_3 were N and amino acid metabolism, secondary metabolism, TCA cycle and minor CHO metabolism and hormone metabolism (as in Figure 4-6, Table 4-3 and discussed in the following sections).

As indicated from these FC studies, the N-regulated gene expression changes in -Rhiz and +Rhiz followed a similar trend in respect to the number of induced or repressed significantly expressed genes at 2 and 6 h time points (Table 4-1). In studying the patterns of gene expression changes in different clusters several clusters also showed similar responses to high N treatment in -Rhiz and +Rhiz at 2 and 6 h. Clusters showing similar patterns between -Rhiz and +Rhiz mainly contained genes involved in N assimilation. For example, a large number of significantly expressed genes in response to high N at 2 and 6-hour time points belonged to cluster 16 (with 511 genes) (Figure 4-5 b). Gene expression levels were similar in -Rhiz and +Rhiz during low N growth (0 h) and similar expression levels were also observed in response to high N treatment at 2 and 6 hour time points.

Rhizobia also had a strong effect on gene expression. A strong rhizobia effect (FC>2 or FC<-2) was observed in 11% and 10% of the differentially expressed genes 2 and 6 hours post N-treatment respectively. This rhizobia effect showed dominant upregulation of genes with 96% and 91% of the

Figure 4-7


N-regulated
(a)
Transport
TCA
Stress
Signaling
Secondary metabolism
Transcriptional regulators
Redox
Regulation of protein activity
N-metabolism
Minor CHO metabolism
Hormone metabolism
Development
Cell
Cell wall
Amino acid metabolism
59% 75% 52% 76%
100% 100% 100% 100%
62% 92% 63% 76%
59% 62% 68% 70%
6% 31% 17% 28%
56% 80% 71% 74%
67% 70% 56%
67% 60% 61% 84%
100% 100% 100% 100%
33% 67% 86% 80%
13% 50% 31% 55%
29% 100% 88% 100%
0% 0% 38% 100%
20% 60% 27% 60%
83% 100% 77% 100%
0 4 8 12 16 20 24 28 32 36 40 44 48
Number of transcripts
+Rhiz 6 h
+Rhiz 2 h
-Rhiz 6 h
-Rhiz 2 h

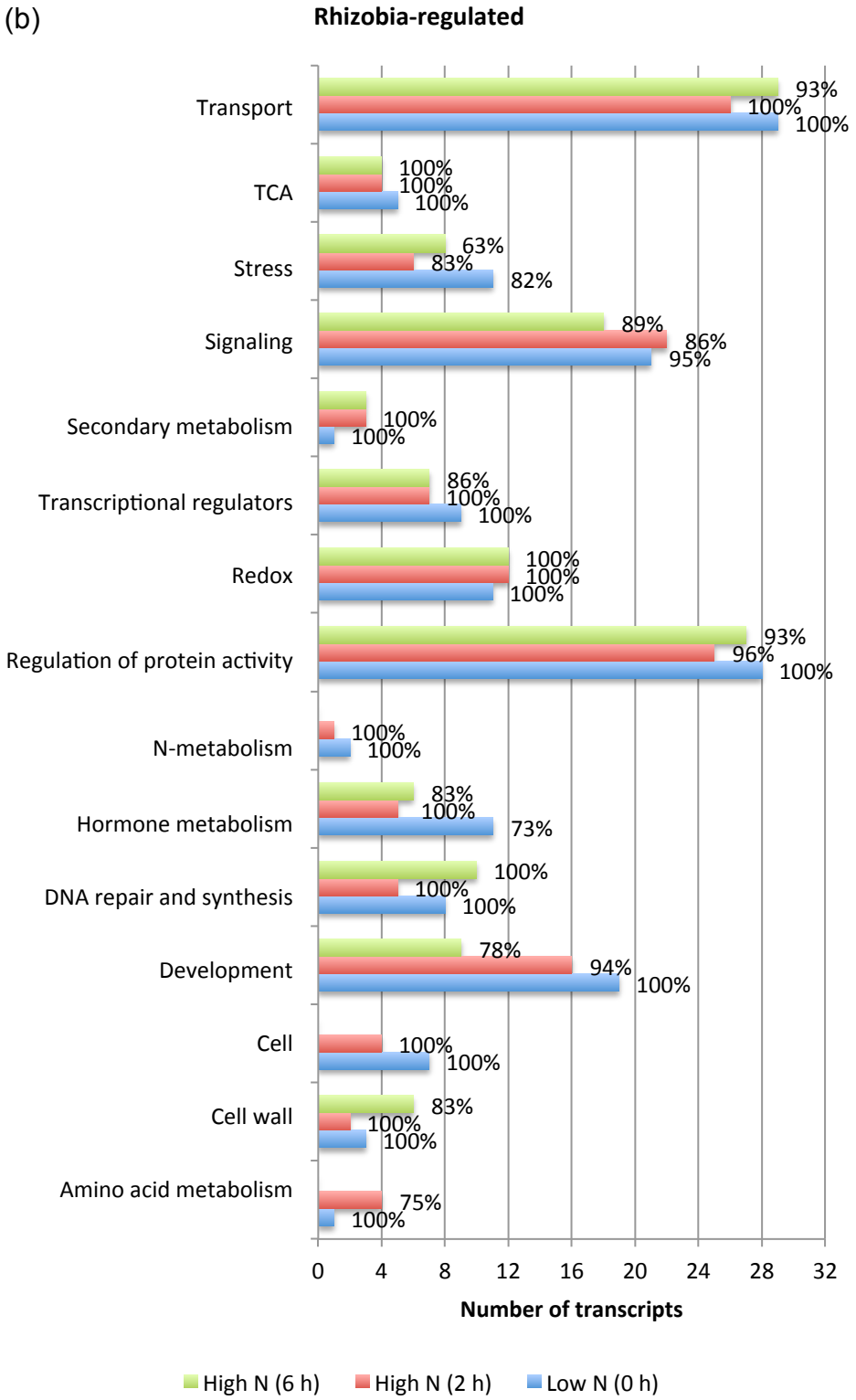


Figure 4-7: Main functional categories defined by MapMan software (Thimm et al., 2004) and the number of (a) N regulated or (b) rhizobia regulated genes (FC >2 or FC <-2) assigned to each category. Nodulins and some of the genes involved in nodulation are not included in these categories. The numbers on top of each bar are the percentage of upregulated (FC>2) genes. -Rhiz= mock-inoculated, +Rhiz= rhizobia inoculated; 2 h and 6 h= 2 and 6 h post treatment with high N; high N= treated with 5 mM N, low N= grown at low N (0.1 mM). Table 4-3 contains Wilcoxon rank sum test P values for each functional category (functional categories with $P > 0.05$ are not included in the table 4-3).

differentially expressed genes being upregulated at 2 and 6-hour time points (Table 4-1). Strong rhizobia effect was also present in seedlings grown at low N (0 hour time point) and 12% of the differentially expressed genes showed strong (FC>2 or FC<-2) gene expression changes (97% upregulated) (Table 4-1).

The differentially expressed genes showing significant (FC>2 or FC<-2) expression changes in response to high N treatment and rhizobia effect (Table 4-1) were further analysed using the MapMan (Thimm et al., 2004) software to assign groups of genes to different pathways and functional categories (Table 4-3 and Figure 4-7). This allowed the interpretation of the data in the context of known biological processes or pathways. The results from this analysis showed that that the main N and rhizobia responses were related to primary N responses, development and regulation of N assimilation and RSA development. Figure 4-7 shows the main functional categories defined by MapMan analysis and the number of N regulated (Figure 4-7 a) or rhizobia regulated (Figure 4-7 b) genes (FC>2 or FC<-2) assigned to each category. Functional categories were selected based on the Wilcoxon rank sum test with Benjamini-Hochberg corrected ($P < 0.05$) (Table 4-3). Some functional categories that did not meet this cutoff ($P < 0.05$) but had important role in N/rhizobia responses were also selected for further study (some of the functional categories in Figure 4-7). Also, MapMan assigned the majority of nodulins and some other genes involved in nodulation to a miscellanies category hence they are not mentioned in the graphs in Figure 4-7. Thus, six different groups were depicted: i) N assimilation and amino acid metabolism; ii) metabolism: tricarboxylic acid cycle (TCA cycle), minor carbohydrate metabolism and secondary metabolism; iii) development: cell wall precursor (synthesis, degradation), genes involved in LR and nodule developmental stages; iv) regulation: TFs, post translation modification of proteins (protein synthesis and degradation), hormones, redox and signaling; v) stress (biotic and abiotic) and vi) transport (such as nitrate and amino acid transport). The vast majority of the gene expression changes were induction of gene

Table 4-4: A selection of significantly regulated (FC>2 or FC<-2) genes that showed the highest gene expression changes in response to high N (2 and 6 h pt) at mock-inoculated (-Rhiz) and rhizobia inoculated (+Rhiz) plants assigned to different functional categories using MapMan (Thimm et al., 2004). Tables are sorted alphabetically by the functional categories column.

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Nitrogen metabolism	nitrate metabolism: nitrite reductase	Medtr4g086020.1	Ferredoxin-nitrite reductase (NIR1)	6.9	6.8	2.5	2.5
	nitrate metabolism: nitrate reductase	Medtr3g073180.1	Nitrate reductase NADH dependent	3.8	4.9	3.6	3.6
	nitrate metabolism: nitrate reductase	Medtr3g073150.1	Nitrate reductase NADH dependent (NR1)		2.4		
	nitrate metabolism: nitrate reductase	Medtr5g059820.1	Nitrate reductase (NR2)	2.1	2.8	2.6	2.5
	N-degradation: glutamate dehydrogenase	Medtr6g029460.1	Glutamate dehydrogenase 1	2.5		2.6	
Amino acid metabolism	degradation: aromatic amino acid, tyrosine	Medtr8g061360.1	Tyrosine aminotransferase	3.4	2.7	2.4	2.1
	degradation: aspartate family, asparagine	Medtr3g102370.1	L-asparaginase	2.1			
	degradation: central amino acid metabolism, aspartate	Medtr6g007070.1	L-aspartate oxidase		-3.6		-2.2
	synthesis: central amino acid metabolism	Medtr5g067370.1	Alanine glyoxylate aminotransferase	3.3	3.3	2.6	2.8
	synthesis: serine-glycine-cysteine group, cysteine	Medtr7g086380.1	2-aminoethanethiol dioxygenase		2.5		
Transport	transport: amino acids	Medtr8g094290.1	Amino acid permease		3.0		2.3
	transport: amino acids	Medtr3g110660.1	Amino acid permease	-2.1			
	transport: nitrate	Medtr2g085510.1	High-affinity nitrate transporter	5.4	5.0	6.0	5.4
	transport: nitrate	AC233663 23.1	High-affinity nitrate transporter	4.4	4.0	5.7	4.7
	transport: nitrate, peptides and oligopeptides	Medtr5g012290.1	Nitrate transporter			2.5	

Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Metabolism	TCA: transformation	Medtr5g066060.3	Carbonic anhydrase	5.4	4.3	3.6	3.1
	TCA: transformation	Medtr5g066060.4	Carbonic anhydrase	5.1	3.8	3.5	2.8
	TCA: transformation	Medtr5g066060.2	Carbonic anhydrase	4.9	3.8	3.4	2.9
	TCA: transformation	Medtr5g066060.1	Carbonic anhydrase	4.9	3.8	3.3	2.6
	minor CHO metabolism: myo-inositol	Medtr8g102150.1	Inositol oxygenase 2	3.1		3.3	
	minor CHO metabolism	Medtr4g108260.1	Aldose 1-epimerase-like protein	3.5			
	minor CHO metabolism	Medtr7g080530.1	Phosphoglycolate phosphatase		-2.2		
	minor CHO metabolism	Medtr4g128840.2	Xylose isomerase	2.5	2.7		
	minor CHO metabolism: raffinose family, raffinose synthases	Medtr3g077280.1	Galactinol--sucrose galactosyltransferase	-2.6	-4.7	-2.1	-3.4

Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Hormone metabolism	auxin (induced, regulated, responsive, activated)	Medtr5g091090.1	SAUR family protein	2.5			
	auxin (induced, regulated, responsive, activated)	Medtr3g084240.1	Auxin-induced protein-like		2.4		
	auxin (signal transduction)	Medtr7g079720.1	Auxin Efflux Carrier		-5.0		-5.1
	auxin (synthesis, degradation)	Medtr2g097530.1	IAA-amino acid hydrolase ILR1-like 4	-2.4	-2.9		
	brassinosteroid (synthesis, degradation, sterols)	Medtr4g092640.1	Squalene epoxidase 1		-2.7		-2.3
	cytokinin (synthesis, degradation)	Medtr3g036100.1	Cytokinin dehydrogenase 1	2.2			
	ethylene (signal transduction)	AC233556 27.1	Ethylene-responsive transcription factor 5	2.5	2.6	2.4	2.1
	ethylene (synthesis, degradation)	Medtr8g009120.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1	2.2			
	ethylene (synthesis, degradation)	Medtr6g092620.1	1-aminocyclopropane-1-carboxylate oxidase	3.3	3.2		
	ethylene (synthesis, degradation)	Medtr8g009130.1	1-aminocyclopropane-1-carboxylate oxidase homolog 4		2.3		
	gibberelin (induced, regulated, responsive, activated)	Medtr7g090590.1	Gibberellin induced protein			-2.9	-3.3
	gibberelin (synthesis, degradation)	Medtr7g090520.2	Gibberellin 20 oxidase 2	-3.8	-3.7	-3.3	-3.6
	gibberelin (synthesis, degradation)	Medtr7g090520.1	Gibberellin 20 oxidase 3	-4.3	-4.2	-3.8	-3.8

Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Hormone metabolism	jasmonate (signal transduction)	Medtr5g013530.1	Protein TIFY 7	-3.0	-2.2		-2.4
	jasmonate (synthesis, degradation, lipoxygenase)	Medtr8g018520.1	Lipoxygenase		-2.4		
	jasmonate (synthesis, degradation, lipoxygenase)	Medtr8g021020.1	Lipoxygenase			2.4	
	jasmonate (synthesis, degradation)	Medtr5g008040.1	12-oxophytodienoate reductase				-2.4
	salicylic acid (synthesis, degradation)	Medtr7g084350.1	Jasmonate O-methyltransferase	2.1		2.2	
	salicylic acid (synthesis, degradation)	Medtr5g020940.1	Jasmonate O-methyltransferase	-3.4	-3.5		
Trascriptional regulators	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr3g098580.1	Ethylene-responsive transcription factor RAP2-6	2.2	2.7		
	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr7g046260.1	Ethylene-responsive transcription factor ERF034	-2.3	-2.2		
	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr2g015050.1	Ethylene responsive transcription factor 1b		2.3		
	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr5g008550.1	Ethylene-responsive transcription factor 4		-2.6		-2.2
	ARF, Auxin Response Factor family	Medtr2g093740.1	Auxin response factor 4		2.4		
	AS2,Lateral Organ Boundaries Gene Family	Medtr4g095600.1	LOB domain-containing protein 38	4.0	3.5	3.3	3.3
	AS2,Lateral Organ Boundaries Gene Family	Medtr5g080470.1	LOB domain-containing protein 33	3.3	2.6		

Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Transcriptional regulators	AS2,Lateral Organ Boundaries Gene Family	Medtr1g106420.1	LOB domain-containing protein 38	3.2	4.3		3.3
	AS2,Lateral Organ Boundaries Gene Family	Medtr5g015880.1	LOB domain-containing protein 39	2.6	2.3	2.0	2.0
	AS2,Lateral Organ Boundaries Gene Family	Medtr4g060950.1	LOB domain protein 11	2.1		2.0	
	Aux/IAA family	Medtr7g110790.1	Auxin-responsive protein IAA4	2.4	2.6		
	bHLH,Basic Helix-Loop-Helix family	Medtr1g106470.1	Transcription factor bHLH84	2.5			
	bHLH,Basic Helix-Loop-Helix family	Medtr3g116770.1	Transcription factor BEE 2	2.4		2.1	
	bHLH,Basic Helix-Loop-Helix family	Medtr7g090410.1	Transcription factor ORG2	2.1	2.4		
	bHLH,Basic Helix-Loop-Helix family	Medtr4g098250.1	Transcription factor bHLH25	-2.4			-2.2
	bHLH,Basic Helix-Loop-Helix family	Medtr3g099620.1	Transcription factor bHLH96			-2.1	-2.4
	bZIP transcription factor family	Medtr7g115120.2	Opaque 2	2.4		2.6	2.1
	bZIP transcription factor family	Medtr7g115120.1	Opaque 2	2.4		2.4	
	bZIP transcription factor family	Medtr5g015090.1	Protein ABSCISIC ACID-INSENSITIVE 5	2.3			
	bZIP transcription factor family	Medtr4g097440.1	Ocs element-binding factor 1		2.5		2.2

Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Transcriptional regulators	bZIP transcription factor family	Medtr5g015090.1	Protein ABSCISIC ACID-INSENSITIVE 5		2.0		
	C2C2	Medtr3g082630.3	Zinc finger protein CONSTANS-LIKE 10				4.3
	C2C2	Medtr3g082630.1	Zinc finger protein CONSTANS-LIKE 9				3.9
	C2C2	Medtr3g082630.2	Zinc finger protein CONSTANS-LIKE 9				3.7
	C2C2	AC233660 33.1	Zinc finger protein CONSTANS-LIKE 16				3.1
	G2-like transcription factor family, GARP	Medtr1g093420.1	Two-component response regulator ARR2	5.0	4.7	5.2	5.2
	G2-like transcription factor family, GARP	Medtr1g093080.1	Two-component response regulator ARR2	3.5	3.3	3.3	3.3
	G2-like transcription factor family, GARP	Medtr5g017980.1	Two-component response regulator ARR1	3.4	3.7	3.9	4.4
	G2-like transcription factor family, GARP	Medtr4g064730.1	Two-component response regulator ARR1		3.1		
	G2-like transcription factor family, GARP	Medtr4g064890.1	Two-component response regulator ARR1		2.9		
	MYB-related transcription factor family	Medtr7g118330.1	Circadian clock-associated protein 1a	-2.2	-4.0	-2.8	-4.8
	Psudo ARR transcription factor family	Medtr3g092780.1	Two-component response regulator-like PRR73			3.3	3.1
	WRKY domain transcription factor family	Medtr7g038380.1	WRKY transcription factor 44	2.2			
	WRKY domain transcription factor family	Medtr7g110720.1	WRKY transcription factor 5	-2.2	-2.2		

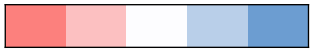
Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Regulation of protein activity	degradation	Medtr2g035370.1	Allantoate amidohydrolase		-2.2		-2.8
	degradation	Medtr3g077430.1	Carboxyl-terminal proteinase		-3.0	-2.0	
	degradation	Medtr2g035370.2	Allantoate amidohydrolase				-2.8
	degradation: aspartate protease	Medtr4g100990.1	Aspartic proteinase nepenthesin-2	2.7	4.0		
	degradation: cysteine protease	Medtr8g086470.1	Cysteine proteinase 2	3.4		2.9	
	degradation: metalloprotease	Medtr5g036090.1	Matrix metalloproteinase-14			-2.4	-3.5
	degradation: ubiquitin E3 BTB/POZ Cullin3 BTB/POZ	Medtr4g104140.2	Speckle-type POZ protein	2.2	2.6		2.5
	degradation: ubiquitin E3 BTB/POZ Cullin3 BTB/POZ	Medtr4g104140.1	Speckle-type POZ protein	2.1	2.3		2.3
	degradation: ubiquitin E3 RING	AC235758 13.1	RING finger protein 44	2.4			
	degradation: ubiquitin E3 RING	Medtr8g073120.1	Ring finger protein		2.9		2.6
	degradation: ubiquitin E3 RING	Medtr8g092910.1	RING finger protein 43		2.1		
	degradation: ubiquitin E3 RING	Medtr8g074900.1	RING-H2 finger protein ATL4M			-2.1	
	degradation: ubiquitin E3 SCF FBOX	Medtr8g085650.1	F-box/kelch-repeat protein At2g44130	2.3	2.4		

Table 4-4, continue

N-regulated responses							
				FC			
				-Rhiz		+Rhiz	
	Functional category	Gene ID	Annotation	2 hr	6 hr	2 hr	6 hr
Regulation of protein activity	degradation: ubiquitin E3 SCF FBOX	Medtr8g062300.1	F-box family-3	-2.2			
	degradation: ubiquitin E3 SCF FBOX	Medtr8g105590.1	Flavin-binding kelch repeat F-box 1		2.5		
	degradation: ubiquitin E3 SCF FBOX	Medtr5g070080.1	F-box		-2.7		
	degradation: ubiquitin E3 SCF FBOX	Medtr8g105590.1	Flavin-binding kelch repeat F-box 1				2.8
	postranslational modification	Medtr1g113960.1	Protein kinase like protein	3.6	3.3	2.3	2.8
	postranslational modification	Medtr5g088350.1	CBL-interacting protein kinase (CIPK)	2.0			2.1
	postranslational modification	Medtr2g088020.1	Mitogen-activated protein kinase kinase kinase A		-2.7		
	postranslational modification kinase receptor like cytoplasmatic kinase VII	Medtr2g028580.2	Receptor protein kinase-like	2.7	3.2		
	postranslational modification kinase receptor like cytoplasmatic kinase VII	Medtr1g040200.1	Kinase-like protein	-2.1			
	postranslational modification kinase receptor like cytoplasmatic kinase VII	Medtr2g028580.2	Receptor protein kinase-like			2.1	2.6
CEP peptides		AC233112_1013.1	MtCEP7	-1.2	-1.8	-0.7	-1.0
		AC233112_1014.1	MtCEP8	-2.0	-1.2	-2.1	-2.6

FC -5.1 6.9



expression by N (-Rhiz or +Rhiz) or rhizobia effect rather than repression as the upregulation trend also in the previous section.

4.2.3.1 N transport, assimilation and amino acid metabolism is rapidly regulated upon N treatment

Treatment of -Rhiz and +Rhiz seedlings to high N for 2 and 6 h after growth under low N (N-depravation) conditions resulted in significant induction (FC>2) of some of the genes involved in N transport and assimilation (Table 4-4). The effect of rhizobia on the expression changes of the genes involved in N transport and assimilation and amino acid metabolism at low N (0 h) and high N treated plants (2 and 6 h) had a quantitatively lower gene expression regulatory effect that the N response (Table 4-5). The N-induced genes could be primary response genes induced during PNR and show a similar response in -Rhiz and +Rhiz. As also seen in *Arabidopsis*, PNR genes are significantly expressed shortly after treatment with nitrate in roots (Krouk et al., 2010b), with *Arabidopsis* pericycle, stele and LR cap the earliest and most responsive tissues to nitrate (Gifford et al., 2008, Krouk et al., 2010b). There are numerous studies indicating that, as a signal molecule, nitrate regulates gene expression and the genes involved in its own assimilation such as genes encoding NR and NiR as well as many transporters of the NRT1 and NRT2 family (Bouguyon et al., 2012). The expression of several genes involved in N transport and assimilation was induced by adding nitrate (and not other N sources) to soil or medium (Stitt, 1999). Nitrate transporters (Lejay et al., 1999), nitrate assimilation enzymes (Gowri et al., 1992) and carbon assimilation enzymes involved in N/carbon balance (Sakakibara et al., 1997, Scheible et al., 1997) are all regulated by nitrate. In the absence of nitrate these genes are expressed at a low level.

Studying the differentially expressed N-regulated genes with significant (FC>2 or FC<-2) expression changes at 2 and 6 hours shows that many of the genes involved in different steps of N assimilation were strongly upregulated in both -Rhiz and +Rhiz (Table 4-4). In response to high N

treatment the differentially expressed high affinity nitrate transporters Medtr2g085510.1 and AC233663_23.1 were highly induced (average FC of 5) and showed a similar expression pattern in -Rhiz and +Rhiz at both 2 and 6 h (Table 4-4). Based on MapMan pathway analysis these two transporters are highly similar to the AtNRT2.1 high affinity transporter. Studies on *Arabidopsis* (Zhuo et al., 1999) have also reported that local supply of nitrate induces the expression of AtNRT2.1. The differentially expressed nitrate transporter Medtr5g012290.1 (highly similar to *AtNRT1.1* based on MapMan pathway analysis) showed significant (FC=2.5) expression changes in response to high N specifically at 2 h in +Rhiz (Table 4-4). The local signaling pathway activated in response to localized high nitrate concentrations affect RSA (Zhang et al., 1999). AtNRT1.1 promotes LR elongation in response to localized high nitrate concentration (Remans et al., 2006a) it is also involved in auxin transport and accumulation required for LR growth (Bouguyon et al., 2012). Here the significant induction of NRT1.1 (Medtr5g012290.1) only in +Rhiz at high N may suggest that its expression pattern is affected by rhizobia and also that it could be one of the components of the regulatory pathway responsible for short PR and LRs at high N in the presence of rhizobia (Figure 4-1).

The significant (FC>2) induction of the nitrate transporters Medtr2g085510.1, AC233663_23.1 and Medtr5g012290.1 that are highly similar to AtNRT2.1 and AtNRT1.1 could be evidence of similar rapid N assimilation in *Medicago.* This could be part of the PNR as also seen in *Arabidopsis* that under short-term supply of nitrate NRT2.1 is upregulated by NRT1.1 (Ho et al., 2009). Under high nitrate concentrations AtNRT1.1 is T101-dephosphorylated and acts as a low affinity transporter. This leads to the full induction of AtNRT2.1 (Ho et al., 2009). This mechanism provides an explanation for the high induction of the two high affinity transporters in response to high N treatment.

High N treatment also caused the strong induction of differentially expressed enzymes involved in the N assimilation pathway such as nitrate

reductases, ferredoxin-nitrite reductase and glutamate dehydrogenase in -Rhiz and +Rhiz. The Ferredoxin-nitrite reductase Medtr4g086020.1 (Highly similar to AtNIR1) was significantly expressed in response to high N in -Rhiz and +Rhiz at 2 and 6-hour time points (Table 4-4). Ferredoxin-nitrite reductase is a key enzyme in the assimilation of N since it catalyzes the six-electron reduction of nitrite to ammonium (Orea et al., 2001). In the non-nodulating (-Rhiz) plants this enzyme showed a more strong induction in response to high N treatment at 2 and 6 h (FC>6) compared to +Rhiz (FC>2). This suggests that once supplied with high N, a stronger N assimilation activity was induced in the -Rhiz plants that had no previous access to N source in the form of N_2. This contrasts to the nodulating plants (+Rhiz) that were able to acquire some of their N requirements through nodulation when under N limiting (low N) conditions.

In contrast to early N assimilation genes, the number of differentially expressed N-regulated genes assigned as enzymes of amino acid metabolism pathways was higher in -Rhiz samples. This may be in agreement with the higher N assimilation activity observed in -Rhiz to compensate for the previous N limitation conditions they were subjected to. These enzymes were mainly upregulated and involved in the degradation such as L-asparaginase, Tyrosine aminotransferase and 3-methylcrotonyl-CoA carboxylase. Some differentially expressed genes annotated as degradation enzymes (e.g. L-aspartate oxidase) were repressed and some annotated as enzymes involved in synthesis of amino acids (e.g. 2-aminoethanethiol dioxygenase) were induced (Table 4-4). In +Rhiz samples, differentially expressed genes assigned as enzymes involved in amino acid synthesis were more than the ones involved in degradation. The differentially expressed genes that were commonly expressed in +Rhiz between both time points were generally upregulated and had a similar expression pattern in both -Rhiz and +Rhiz. For example alanine glyoxylate aminotransferases that participate in alanine synthesis were strongly induced in both time points in both -Rhiz and +Rhiz. At 2 h, all the significantly expressed enzymes involved in amino acid metabolisms were induced in -Rhiz and +Rhiz. This may be part of the early

response to N as an induction of assimilation activities. At the later time point (6 h) some repression were observed among the amino acid metabolism enzymes in -Rhiz and +Rhiz suggesting balancing mechanism for amino acid metabolism.

4.2.3.2 Regulation of carbon metabolism during N treatment and nodulation

There was a strong response (FC>2 or FC<-2) to N among differentially expressed TCA cycle enzymes and proteins involved in carbohydrate metabolism at 2 and 6 h in both nodulating and non-nodulating plants (Table 4-4). This may be to provide the necessary carbon and energy pool for the enhanced metabolic and developmental activities due to the availability of N. This fits with previous studies in *Arabidopsis* that suggest that carbon assimilation enzymes involved in N/carbon balance (Sakakibara et al., 1997, Scheible et al., 1997) are regulated by nitrate. Four carbonic anhydrases (Medtr5g066060) involved in the TCA cycle were differentially expressed (2 < FC < 5) in response to high N in nodulating and non-nodulating plants with a higher expression at 2 h (Table 4-4). Carbonic anhydrases are some of the key enzymes in CO_2 metabolism, catalyzing the reversible hydration of CO_2 to form bicarbonate. The higher overexpression of these genes in -Rhiz compared to +Rhiz and the lower number of significantly expressed genes involved in the metabolism of minor carbohydrates (with some strongly repressed) in +Rhiz suggests that a stronger carbon metabolism activity is triggered in non-nodulating plants once supplied with high N. This could be to provide enough carbon resources for the increased developmental processes when enough N resources are available. This could be one of the reasons that +Rhiz has shorter LRs in high N compared to -Rhiz in the phenotypic analysis of plant responses to N (Figure 4-1). Enzymes involved in the metabolism of minor carbohydrates were mainly induced at 2 and 6 hour pt. Although, fewer enzymes showed significant expression in +Rhiz compared to -Rhiz at both time points and some were strongly repressed (Table 4-4).

Table 4-5: A selection of significantly regulated (FC>2 or FC<-2) genes that showed the highest gene expression changes in response to rhizobia mediated low (0 h) or high (2 and 6 h) N response assigned to different functional categories using MapMan (Thimm et al., 2004). Tables are sorted alphabetically by the functional categories column.

Rhizobia-regulated responses						
	Functional category	Gene ID	Annotation	FC		
				0 hr	2 hr	6 hr
Nodulins		Medtr1g030270.2	Early nodule-specific protein	3.9	4.6	4.3
		Medtr1g030270.3	Early nodule-specific protein	6.2	5.7	6.2
		Medtr4g130780.1	Early nodulin	7.4	7.9	7.6
		Medtr1g030270.1	Early nodulin	6.1	5.3	5.4
		Medtr7g065770.2	Early nodulin ENOD18	3.7	4.3	2.4
		Medtr4g130800.1	Early nodulin-20	3.1	5.1	4.3
		Medtr6g044700.1	Late nodulin	4.9	5.6	4.7
		AC146565_34.1	MtN11 protein	5.0	4.0	2.3
		Medtr2g030470.1	MtN19 protein	2.5	3.4	3.0
		Medtr7g086040.1	MtN20 protein	4.6	5.9	5.0
		Medtr7g114890.1	MtN26 protein	2.4	2.6	2.7
		Medtr6g089330.1	MtN28 protein	4.4	4.4	2.4
		Medtr7g071720.2	Nodule-specific cysteine-rich peptide 122	6.8	8.6	6.2
		Medtr7g008940.1	Nodule-specific cysteine-rich peptide 147	5.9	7.5	6.4

Table 4-5, continue

Rhizobia-regulated responses						
	Functional category	Gene ID	Annotation	FC 0 hr	FC 2 hr	FC 6 hr
Nodulins		Medtr6g045150.1	Nodule-specific cysteine-rich peptide 172	7.2	8.4	7.7
		Medtr4g065310.1	Nodule-specific cysteine-rich peptide 206	5.6	7.8	6.3
		Medtr4g031380.1	Nodule-specific cysteine-rich peptide 319	5.1	7.4	7.2
		Medtr5g056890.1	Nodule-specific cysteine-rich peptide 88	6.4	7.5	5.9
		Medtr5g084260.1	Nodule-specific glycine-rich protein 1L	6.6	7.7	5.7
		Medtr3g055440.2	Nodulin 25	6.0	8.1	6.5
Redox	Heme	Medtr1g011540.1	Leghemoglobin	6.6	7.9	6.6
		Medtr5g081000.1	Leghemoglobin	5.6	6.7	4.6
		Medtr5g081030.1	Leghemoglobin	5.0	7.4	4.8
		Medtr5g066070.2	Leghemoglobin B	4.8	5.2	4.0
		Medtr5g041610.2	Leghemoglobin K	4.9	5.3	3.7
Cell wall degradation	pectate lyases and polygalacturonases	Medtr3g070740.1	Pectate lyase	2.6		
	pectate lyases and polygalacturonases	Medtr2g032710.1	Polygalacturonase	2.9	3.6	
	pectate lyases and polygalacturonases	Medtr8g006500.1	Polygalacturonase			-2.1
	pectate lyases and polygalacturonases	Medtr3g111410.1	Rhamnogalacturonate lyase			2.2

Table 4-5, continue

Rhizobia-regulated responses						
				FC		
	Functional category	Gene ID	Annotation	0 hr	2 hr	6 hr
Metabolism	TCA / org. transformation.carbonic anhydrases	Medtr3g077910.1	Carbonic anhydrase	5.3	4.3	4.0
	TCA / org. transformation.carbonic anhydrases	Medtr3g077910.2	Carbonic anhydrase	5.5	4.3	4.1
	TCA / org. transformation.carbonic anhydrases	Medtr3g077910.3	Carbonic anhydrase	3.1	2.8	2.1
	TCA / org. transformation.carbonic anhydrases	Medtr3g077940.1	Carbonic anhydrase	3.4		
N metabolism	nitrate metabolism	Medtr3g073180.1	Nitrate reductase	2.3	2.1	
	nitrate metabolism: nitrite reductase	Medtr4g086020.1	Ferredoxin-nitrite reductase	4.5		
Amino acid metabolism	synthesis: serine-glycine-cysteine group,cysteine	AC231371 20.1	2-aminoethanethiol dioxygenase	2.3		
	synthesis: serine-glycine-cysteine group,cysteine	Medtr7g086380.1	2-aminoethanethiol dioxygenase	3.9	3.3	
Transport	transport.amino acids	Medtr8g089360.1	High affinity cationic amino acid transporter 1	5.8	5.6	4.3
	transport.ammonium	Medtr7g098930.1	Ammonium transporter 1 member 4			2.4
	transport.peptides and oligopeptides	AC229701 3.1	Oligopeptide transporter OPT family	3.6	3.1	2.5
	transport.peptides and oligopeptides	Medtr1g116930.1	Peptide transporter PTR1	4.8	4.6	4.4
	transport.peptides and oligopeptides	Medtr7g088790.1	Peptide transporter PTR5	2.1		2.4
	transport.peptides and oligopeptides	Medtr7g092230.1	Oligopeptide transporter OPT family	4.2	4.3	2.8

Table 4-5, continue

Rhizobia-regulated responses						
				FC		
	Functional category	Gene ID	Annotation	0 hr	2 hr	6 hr
Transport	transport.peptides and oligopeptides	Medtr7g092240.1	Oligopeptide transporter OPT family	2.1	2.2	
	transport.peptides and oligopeptides	Medtr7g098160.1	Peptide transporter PTR3-A	2.8		
	transport.peptides and oligopeptides	Medtr7g098220.1	Peptide transporter PTR3-A	5.1	4.4	3.9
	transport.potassium	Medtr8g107510.1	Potassium transporter 25		2.6	2.3
	transport.sugars	Medtr1g104780.1	Hexose transporter	3.4	2.5	2.3
	transport.sugars	Medtr5g077580.1	Inositol transporter 4	2.0	3.4	2.7
	transport.sugars	Medtr6g006140.1	Solute carrier family 2, facilitated glucose transporter member 2	6.5	6.5	8.1
	transport.sugars	Medtr6g006260.1	Sugar transporter family protein	4.1	4.3	4.5
Hormone metabolism	hormone metabolism: auxin induced-regulated-responsive-activated	Medtr3g109160.1	Auxin-induced protein 6B		2.1	3.1
	hormone metabolism: auxin induced-regulated-responsive-activated	Medtr4g072980.1	Auxin-induced SAUR-like protein	3.4	3.7	2.4
	hormone metabolism: auxin induced-regulated-responsive-activated	Medtr8g022340.1	SAUR family protein	3.5		
	hormone metabolism: auxin synthesis-degradation	Medtr2g097530.1	IAA-amino acid hydrolase ILR1-like 4	-2.9		
	hormone metabolism: ethylene signal transduction	Medtr6g010810.1	Unknown Protein	2.1		2.2
	hormone metabolism: ethylene synthesis-degradation	Medtr6g092620.1	1-aminocyclopropane-1-carboxylate oxidase	2.6		

Table 4-5, continue

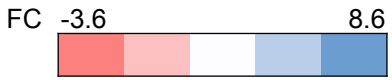
Rhizobia-regulated responses						
				FC		
	Functional category	Gene ID	Annotation	0 hr	2 hr	6 hr
Hormone metabolism	hormone metabolism: gibberelin induced-regulated-responsive-activated	AC235748 1016.1	Snakin-1	2.1		
	hormone metabolism: gibberelin induced-regulated-responsive-activated	Medtr7g090590.1	Gibberellin induced protein	4.0		
	hormone metabolism: jasmonate signal transduction	Medtr5g013530.1	Protein TIFY 7	-2.1		-2.3
	hormone metabolism: jasmonate synthesis-degradation	Medtr5g008040.1	12-oxophytodienoate reductase	3.3	3.4	
	hormone metabolism: salicylic acid synthesis-degradation	Medtr5g020940.1	Jasmonate O-methyltransferase	-2.2		
	hormone metabolism: salicylic acid synthesis-degradation	Medtr5g093900.1	S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase-like protein		2.1	2.6
	hormone metabolism: salicylic acid synthesis-degradation	Medtr7g080940.1	Uncharacterized UDP-glucosyltransferase At1g05670	4.2	4.9	4.4
Transcriptional regulators	AS2, Lateral Organ Boundaries Gene Family	Medtr6g018270.1	LOB domain-containing protein 41		2.1	2.3
	Aux/IAA family	Medtr7g110790.1	Auxin-responsive protein IAA4			-2.2
	bHLH, Basic Helix-Loop-Helix family	Medtr3g099620.1	Transcription factor bHLH96	2.3		
	bHLH, Basic Helix-Loop-Helix family	Medtr4g092700.1	Transcription factor bHLH25	2.1		2.4
	bZIP transcription factor family	Medtr5g015090.1	Protein ABSCISIC ACID-INSENSITIVE 5	2.8		
	C2H2 zinc finger family	Medtr7g082260.1	Zinc finger protein 6		2.8	
	CCAAT box binding factor family, HAP2	Medtr1g056530.1	Nuclear transcription factor Y subunit A-10	3.8	4.9	3.7

Table 4-5, continue

Rhizobia-regulated responses						
				FC		
	Functional category	Gene ID	Annotation	0 hr	2 hr	6 hr
Transcriptional regulators	MYB domain transcription factor family	Medtr4g082230.1	MYB transcription factor	2.2		
	NIN-like bZIP-related family	Medtr5g099060.1	Nodule inception protein	3.5	2.9	2.9
	NIN-like bZIP-related family	Medtr4g068000.1	Nodule inception protein	3.3	3.6	3.1
Regulation of protein activity	degradation, cysteine protease	Medtr5g022560.1	Cysteine proteinase	5.6	6.6	**7.4**
	degradation, cysteine protease	AC233675 19.1	Cysteine protease 5	5.4	6.1	**7.3**
	degradation, cysteine protease	Medtr4g107930.1	Cysteine proteinase	5.1	4.4	5.7
	degradation, cysteine protease	Medtr4g079800.1	Cysteine protease 5	4.9	5.9	**6.8**
	degradation, cysteine protease	Medtr3g044270.1	Cysteine proteinase		3.0	3.8
	degradation, cysteine protease	Medtr4g080730.1	Cysteine proteinase		2.7	3.6
Signaling	signalling.calcium	Medtr3g055570.1	Calmodulin-like protein 1	6.1	6.9	6.6
	signalling.calcium	Medtr3g055570.2	Calmodulin-like protein 2	5.5	6.4	6.0
	signalling.calcium	Medtr3g055520.1	Calmodulin-like protein 3	5.4	7.4	6.7
	signalling.calcium	Medtr3g055510.1	Calmodulin-like protein 5	5.0	5.9	4.5
	signalling.calcium	Medtr3g055480.1	Calmodulin-like protein 6b	3.8	4.5	2.9

Table 4-5, continue

Rhizobia-regulated responses						
	Functional category	Gene ID	Annotation	FC 0 hr	FC 2 hr	FC 6 hr
Signaling	signalling.calcium	Medtr3g055490.1	Calmodulin-like protein 4	2.5		
	signalling.calcium	Medtr6g023460.1	Calcium-binding protein CML38	-3.6		
	signalling.calcium	Medtr6g023460.1	Calcium-binding protein CML38			-2.6
	signalling.receptor kinases.DUF 26	AC235673 7.1	Cysteine-rich receptor-like protein kinase	3.6	3.7	2.4
	signalling.receptor kinases.DUF 26	Medtr4g126930.1	Cysteine-rich receptor-like protein kinase 41		2.4	
	signalling.receptor kinases.leucine rich repeat III	Medtr5g055470.1	Leucine-rich repeat receptor-like protein kinase		2.0	
CEP peptides		AC233112_1013.1	MtCEP7	-0.1	0.4	0.7
		AC233112_1014.1	MtCEP8	-0.1	-0.2	-1.4



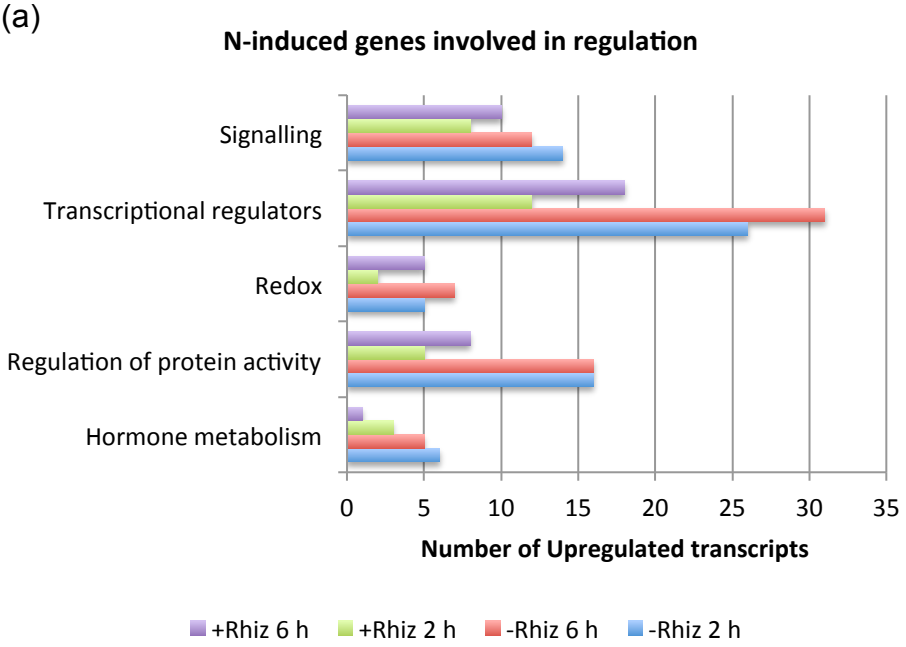
Two carbonic anhydrases, involved in the TCA cycle showed a higher expression change at low N compared to 2 and 6 h high N treatments (Table 4-5). Medtr3g077910 was strongly affected by rhizobia at low N i.e. post treatment with high N (Table 4-5) and Medtr3g077940 was differentially expressed (FC>3) only in low N. The rhizobial regulation of these genes could be related to the level of nodule development and functioning in the plants in response to low and high N concentrations. The expression of some carbonic anhydrases may be controlled by the presence of the rhizobia inside the nodule (Peña et al., 1997). These carbonic anhydrases may be involved in pH regulation and/or CO_2/HCO_3-transport during nodule initiation (Peña et al., 1997) suggesting that they may have different roles at several stages of nodule development and function. The induction response of transporters of N assimilation products, amino acid and peptide transporters affected by rhizobia at low N (Table 4-5) could be an indication of nodule activity at low N condition. Changes in gene expression of carbon metabolism genes in the nodules have been also reported in other studies (El Yahyaoui et al., 2004, Cabeza et al., 2014). Plants provide the bacteroids with the organic carbon from the photosynthesis. This makes the nodules strong carbon sinks and the availability of photoassimilates is one of the important factors controlling nodulation. The energy and carbon skeleton are required for N_2 reduction, assimilation of ammonia and export of nitrogenous compounds.

4.2.3.3 Identification of putative regulatory genes involved in Rhizobia and N responses

N regulation (predominantly as N-induction) and rhizobia regulation was identified for TFs, proteins with signaling functions such as receptor kinases and enzymes involved in post translation modification of proteins (protein synthesis and degradation). Around 22% of the differentially expressed (FC>2 or FC<-2) genes were assigned as regulation genes (Figure 4-8) by the MapMan software (Thimm et al., 2004). These genes (Table 4-4) may be members of the signaling cascade between N sensing and physiological responses to N. Gene expression studies at early time points (up to 20 min)

Figure 4-8

(a)


N-induced genes involved in regulation
Signalling
Transcriptional regulators
Redox
Regulation of protein activity
Hormone metabolism
0 5 10 15 20 25 30 35
Number of Upregulated transcripts
+Rhiz 6 h
+Rhiz 2 h
-Rhiz 6 h
-Rhiz 2 h

(b)

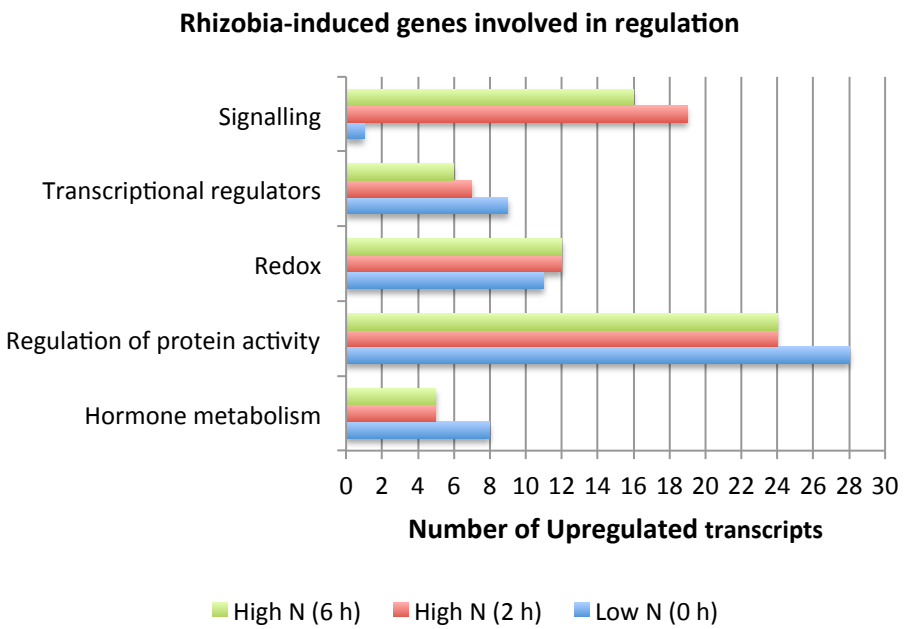

Rhizobia-induced genes involved in regulation
Signalling
Transcriptional regulators
Redox
Regulation of protein activity
Hormone metabolism
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Number of Upregulated transcripts
High N (6 h)
High N (2 h)
Low N (0 h)

Figure 4-8, continue

(c)

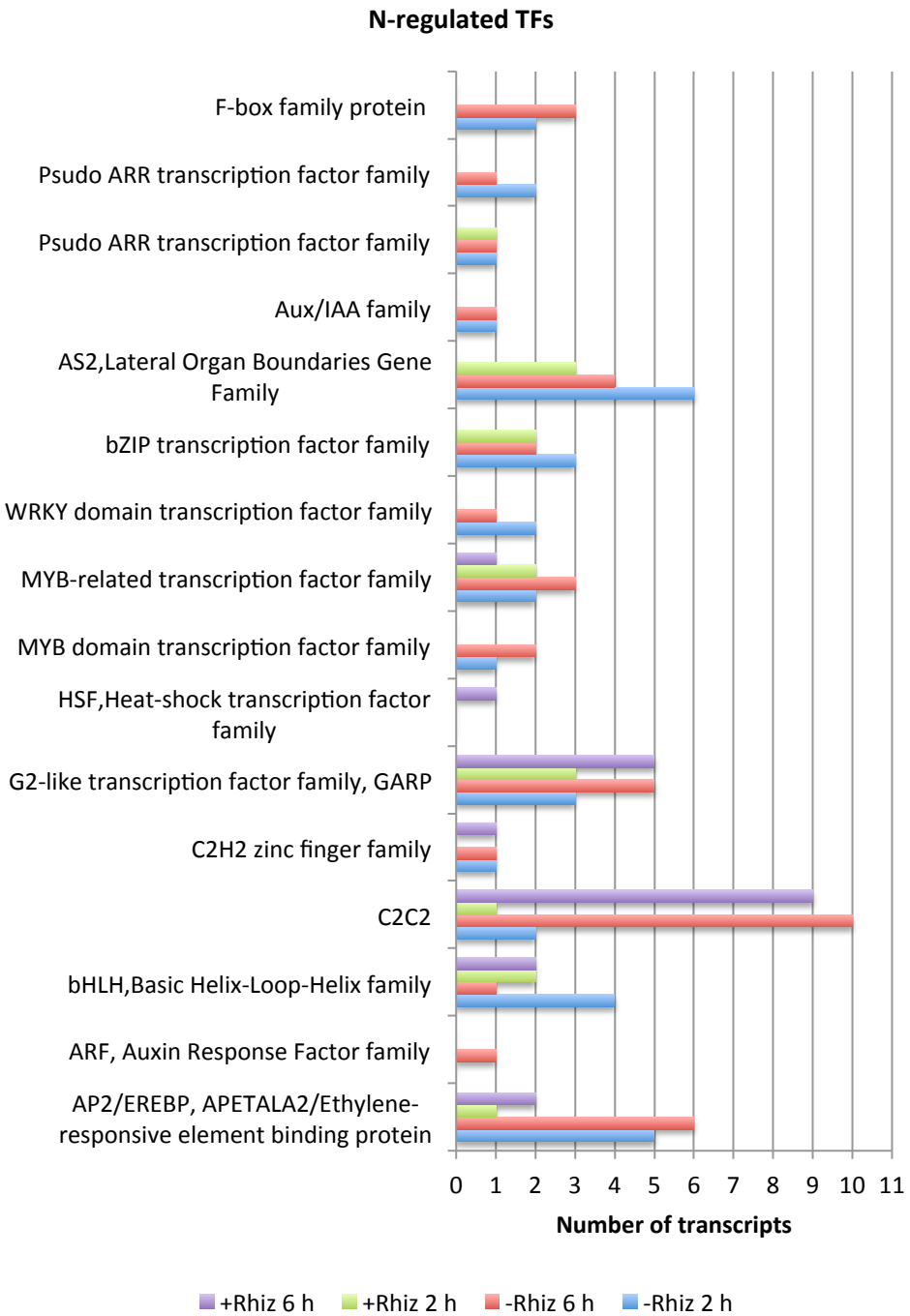
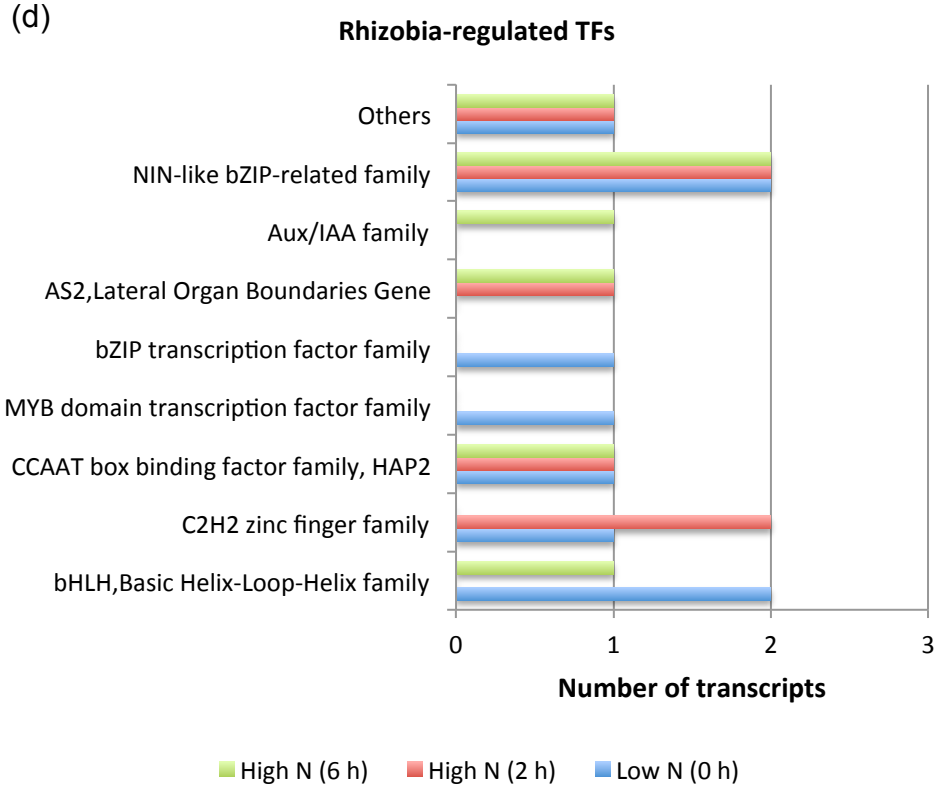
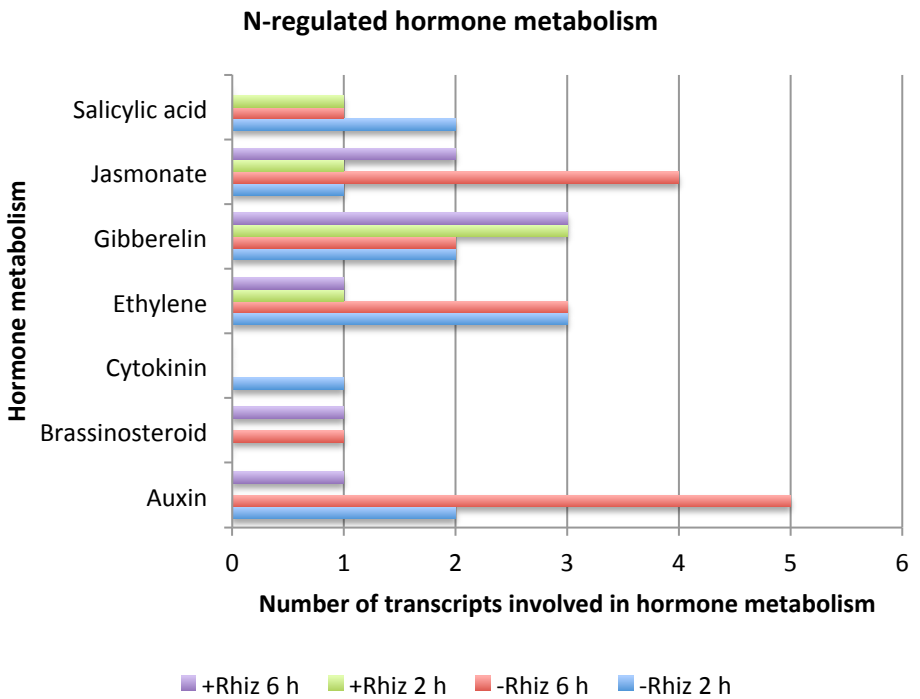


Figure 4-8, continue

(d)



(e)



(f)

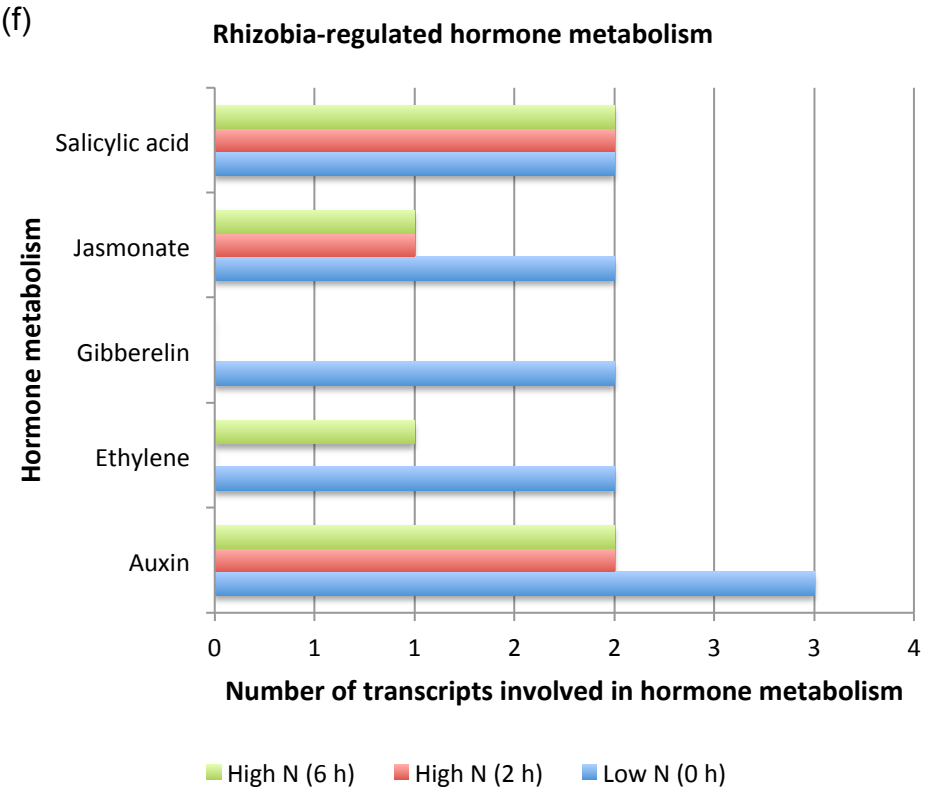


Figure 4-8: Functional classification of putative regulatory genes involved in rhizobia and N responses (FC>2 or FC<-2). (a) N-induced regulatory transcripts, (c) N-responsive TFs and (e) N-regulated transcripts involved in hormone metabolism at 2 and 6 h post treatment with high N in mock inoculated (-Rhiz) and/or rhizobia inoculated (+Rhiz) plants; (b) rhizobia induced regulatory transcripts, (d) rhizobia responsive TFs and (f) rhizobia-regulated transcripts involved in hormone metabolism at low N (0 h) and high N (2 and 6 h).

after exposure to nitrate in *Arabidopsis* suggests that the initial response to nitrate includes genes involved in translation (protein synthesis) followed by regulation of metabolism and hormonal pathways that mediate development (Krouk et al., 2010b). In N-deprived *Arabidopsis*, NO_3^- compared to NH_4^+ treatment resulted in the distinct phosphorylation patterns of proteins involved in regulation including signaling functions, transcriptional regulators and hormone metabolism (Engelsberger and Schulze, 2011).

4.2.3.3.1 Transcriptional regulators

The expression levels of the TFs and their N or rhizobia induction or repression suggest that different groups of TFs in each TF family were responsible for different regulatory responses. Around 8% (in -Rhiz) and 7% (in +Rhiz) of the differentially expressed N-regulated genes (FC>2 or FC<-2) were TFs. N-induction was the prominent response of different groups of TFs (Figure 4-8 c) possibly involved in regulating gene expression responses to high N (2 and 6 h) in -Rhiz and +Rhiz. This made up for 74% and 72% of the TFs in -Rhiz and 80% and 50% in +Rhiz at 2 and 6 h of high N treatment respectively. Among the differentially expressed rhizobia regulated genes with significant expression changes (FC>2 or FC<-2) only 2% were TFs in low N and 1% in high N (2 and 6 h) treated samples. All of these TFs were rhizobia-induced except for one that was suppressed at 6 h high N treatment (Table 4-5).

Different N-regulated members of each TF family usually didn't show the same response between -Rhiz and +Rhiz and/or different time points. For example a higher number of differentially expressed TFs from the APETALA2/Ethylene-responsive element binding protein family (AP2/EREBP) showed strong N response (FC>2 or FC<-2) at -Rhiz (at 2 and 6 h) compared to +Rhiz or differentially expressed members of the WRKY domain transcription factor family showed significant expression changes only in -Rhiz (2 and 6 h), indicating that different TFs were responsible for the N-regulated responses at different time points (2 and 6) and also between -Rhiz and

+Rhiz. This was also observed among rhizobia-regulated TFs and different groups of TFs were responding to low and high N conditions.

The WRKY TFs are involved in the transcriptional regulations during development, metabolism and responses to biotic and abiotic stress but their role in N responses is not yet well known (Canales et al., 2014, Rushton et al., 2010). In this study WRKY TFs showed an N response that was only observed in -Rhiz and rhizobia had no significant effect on the expression changes of WRKY TFs at low or high N either. Two differentially expressed WRKY TFs showed significant N-regulated response only in -Rhiz. Medtr7g038380.1 (WRKY 44) was strongly N-induced (FC>2) in -Rhiz at 2 h and Medtr7g110720.1 (WRKY 5) was N-suppressed (FC<-2) at 2 and 6 h. This suggests a role for WRKY TFs in N responses in the absence of rhizobia that could be related to abiotic stress due to N deficiency in the non-nodulating plants. Another explanation could be that these two WRKY TFs may be involved in the development of LRs and may be one of the reasons for the difference in root phenotype between -Rhiz and +Rhiz at high N (Figure 4-1).

4.2.3.3.1.1 Regulation of N assimilation by circadian rhythms

Of the N-regulated TFs the circadian clock-associated protein 1a (Medtr8g077420.1) from the MYB-related TF family was strongly repressed in all time points in -Rhiz and +Rhiz and showed a rhizobia independent response that was affected by the duration of high N treatment. The expression level of this TF showed variation between time points and the expression level had a two times decrease at 6 h compared to 2 h in -Rhiz and +Rhiz (Table 4-4). In fact, this TF was among the N-suppressed genes showing the highest decrease (FC<-4) in gene expression level at 6 h both in -Rhiz and +Rhiz. In *Arabidopsis* circadian clock-associated protein 1a (CCA1) is one of the main regulators of the circadian clock (McClung, 2006) and is involved in circadian regulation of N assimilation in plants (Gutiérrez et al., 2008). A model has been proposed in *Arabidopsis* (Gutiérrez et al., 2008)

suggesting an interaction between the circadian clock and N-assimilatory pathway. The direct regulatory role of CCA1 on N-assimilation is one of the factors responsible for the clock's affect on the N-assimilatory pathway and the downstream metabolites of the N-assimilation pathway such as Glutamate, Glutamine and other N-metabolites influences the circadian clock by regulating *CCA1* (Gutiérrez et al., 2008). The circadian clock regulates some of the steps in N metabolism such as the expression and activity of nitrate reductases (Gutiérrez et al., 2008). The high affinity nitrate transporters NRT1.1 and NRT2.1 are also coordinately regulated by nitrate and circadian rhythms (Krouk et al., 2010a). In the current study, as N-deprived plants were subject to high N, a PNR starts with upregulation of high affinity nitrate transporters (NRT1.1 and NRT2.1) and nitrate reductases that may be indicative of enhanced N-assimilation activities when N resource is not limiting. The strong N-suppression (FC<-2) of the differentially expressed circadian clock-associated protein 1a TF in response to high N treatment may be indicative of the regulatory response and interaction between the circadian rhythm and N-assimilation.

4.2.3.3.1.2 A putative role for some bHLH TFs in regulating development

The bHLH family are a large family of TFs involved in various signaling and developmental processes such as hormone signaling, symbiotic ammonium transport and root development (Carretero-Paulet et al., 2010) and references within. This TF family is not well studied in the legumes but members have been characterized that participate in symbiotic interactions such as bHLH1 that is involved in controlling nodule vasculature patterning and nutrient exchanges between nodules and roots (Godiard et al., 2011). Here we have identified five differentially expressed N-responsive bHLH TFs (Table 4-4) with significant expression changes (FC>2 or FC<-2) under high N treatment. These TFs showed different responses in -Rhiz and +Rhiz and/or at 2 and 6 h high N treatment. For example Medtr4g098250.1 (bHLH 25) was N-suppressed (FC< 2) at 2 h in -Rhiz and 6 h in +Rhiz and Medtr3g099620.1 (bHLH 96) only in +Rhiz at 2 and 6 h. bHLH 96 along with Medtr4g092700.1

(bHLH 25) were the only bHLH TFs that were significantly (FC>2) affected by rhizobia at low or high N (Table 4-5). These results suggest that bHLH 96 (Medtr3g099620) and bHLH 25 could be involved in N responses and also rhizobia affected responses in the root as they are suppressed in the N-regulated responses and repressed in the rhizobia-regulated ones. It appears that the significant expression change of bHLH 96 is related to the presence of rhizobia and possibly nodulation. The N-suppression of bHLH 96 at high N (2 and 6 h) could be related to the presence of rhizobia or nodulation since it is significantly (FC<-2) down regulated only in the presence of rhizobia irrespective of the N concentration. The rhizobia-induction of this TF is only observed at low N conditions where the plants are able to nodulate. Taking all these together it could be assumed that the regulatory role of bHLH 96 could be dependent on the presence of rhizobia or nodulation activity of the plant and it may be related to controlling developmental activities such as nodulation (as it is induced at low N and had no significant expression changes at high N where nodulation was inhibited) or LR development (as our phenotypic studies showed that LR length could be affected by the presence of rhizobia at high N).

4.2.3.3.2 Hormone metabolism

The expression change of the genes involved in hormone metabolisms shows that N-regulation (predominantly as suppression) of hormone metabolism was stronger at 6 h especially in -Rhiz. The majority of the differentially expressed N-regulated genes (FC>2 or FC<-2) involved in hormonal metabolism were strongly repressed (FC< 2) both in -Rhiz and +Rhiz, particularly at 6 hours of N treatment (Table 4-4); in -Rhiz 69% and in +Rhiz 88% of the genes were downregulated. These were genes that participated in the biosynthesis or signaling pathways of phytohormones auxin, brassinosteroid, ethylene, gibberellin, jasmonate acid (JA) and salicylic acid (SA) (Figure 4-8 e). The expression patterns of the genes were mostly different between -Rhiz and +Rhiz and between time points suggesting that the presence or absence of rhizobia and the duration of high N treatment

were factors affecting hormone metabolism. This could be due to the roles of these hormones in regulating LR development or nodulation in response to N and the cross talk between some of these hormones in regulating these developmental pathways. Auxin is a mobile signal that originates in the shoot and is involved in the systemic regulation of LR development (Alvarez et al., 2012). Other phytohormones are also involved in regulating this process usually in an auxin-dependent way. Auxin distribution that is key in root development is regulated through the special distribution of certain transporters such as PIN family auxin transporters (Saini et al., 2013). Hormones such as JA, SA and GA are involved in regulating the special distribution of PIN family transporters (Sun et al., 2011, Armengot et al., 2014).

The significant (FC>2 or FC<-2) N-regulated expression of some of the genes involved in the JA pathway (Table 4-4) was affected by the presence or absence of rhizobia (Table 4-4). The response of these genes to high N that is also affected by rhizobia may suggest that they could be responsible for the short LR phenotype observed at high N in +Rhiz. JA is involved in plant responses to biotic and abiotic stress as well as development (Wasternack, 2014). It regulates LR formation in *Arabidopsis* through interaction with auxin (Sun et al., 2011, Sun et al., 2009). The subcellular distribution of the PIN family auxin transporters is crucial in auxin gradient-mediated formation of LRs. JA down regulates PIN1 and PIN2 protein levels at the plasma membrane thus negatively regulates auxin transport (Sun et al., 2011, Sun et al., 2009). In *Arabidopsis* ERF109 integrates JA signalling into auxin pathway to regulate LR formation (Cai et al., 2014).

The rhizobia-mediated significant (FC>2 or FC<-2) expression changes of Medtr5g013530.1 (Protein TIFY 7) and Medtr5g008040.1 (12-oxophytodienoate reductase) that are involved in JA pathway (Table 4-5) were not dependent on N concentration. These genes could be involved in the regulation of nodule development through JA. JA is a negative regulator of root-nodule symbiosis (Nagata and Suzuki, 2014, Kinkema and Gresshoff,

2008, Seo et al., 2007, Nakagawa and Kawaguchi, 2006, Sun et al., 2006). In *M. truncatula* NF signaling was inhibited by the application of JA (Zhang et al., 2012, Sun et al., 2006). Although studies also suggest that over a certain range of concentration JA could be a positive regulator of nodulation (Suzuki et al., 2011, Zdyb et al., 2011).

SA is a regulatory signal involved in plant responses to abiotic stress such as drought and salt tolerance (Palma et al., 2013, Chini et al., 2004). It is also involved in plant pathogenic responses by inducing the plant systemic resistance (An and Mou, 2011, Blilou et al., 1999). SA can strongly inhibit nodulation and nodule development (van Spronsen et al., 2003, Stacey et al., 2006). The SA-dependent mechanism in legumes in response to rhizobia is well studied (Robledo et al., 2011, Stacey et al., 2006). The significant (FC>2 or FC<-2) rhizobia-mediated expression changes of three genes involved in the SA pathway (Table 4-5) could suggest that they may be involved in this mechanism. Medtr5g020940.1 (Jasmonate O-methyltransferase) was significantly (FC<-2) suppression at low N (Table 4-5) that could be due to its role in inhibition of SA-mediated control of nodulation. Medtr5g093900.1 (S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase-like protein) was significantly (FC>2) rhizobia-induced only at high N and the significant (FC>2) induction of Medtr7g080940.1 (Uncharacterized UDP-glucosyltransferase At1g05670) was N independent (Table 4-5). The NFs produced by the compatible rhizobia are involved in the inhibition of SA-dependent defense in Legumes (Martinez-Abarca et al., 1998). Inoculation of *Medicago sativa* with compatible rhizobia resulted in decrease of SA level while inoculation with incompatible rhizobia lead to SA accumulation in the root (Martinez-Abarca et al., 1998). The suppression of SA-dependent defence mechanism against compatible rhizobia allows the establishment of the symbiosis (Blilou et al., 1999). Studies also suggest that SA is directly involved in signal transmission in the autoregulation of nodulation (van Spronsen et al., 2003, Sato et al., 2002).

The N-regulated significant expression changes of two Jasmonate O-methyltransferases involved in salicylic acid (synthesis, degradation) pathway (Table 4-4) was independent of presence or absence of rhizobia and their significant (FC>2 or FC<-2) expression changes could be related to the role of SA in root development. Recent studies suggest a role for SA in root development by affecting the endocytic traffic of PIN family auxin-efflux transporters (Armengot et al., 2014, Du et al., 2013). High levels of SA affects PIN1 and PIN2 internalization resulting in the increase of these proteins in plasma membrane (Du et al., 2013). These results suggest that SA and auxin together regulate the clathrin-dependent endocytic mechanism that regulates PIN trafficking and auxin flux and distribution (Armengot et al., 2014, Du et al., 2013).

GA is involved in regulating different aspects of plant growth and development. To our knowledge, no major role for GA in regulating LR development in response to N has been reported. The cross-talk between auxin and GA modulates LR development (Gou et al., 2010). There are studies indicating that GA is a negative regular of LR development. In transgenic *Populus* plants alteration of LR development by GA is partly related to changes in polar auxin transport (Gou et al., 2010). GA mediates auxin transport by regulating the abundance of some of the PIN transporters . Gibberellin 20 oxidase 2 and 3 that are involved in gibberellin biosynthesis (Plackett et al., 2012) were strongly N-repressed (FC<-3, among the most highly N-suppressed genes) at all time points with almost no variation in their expression levels between -Rhiz and +Rhiz (Table 4-4). Indicating that the strong N-suppression of these genes was related to the high N treatment rather than presence or absence of rhizobia and possibly associated with GA role in regulating LR development under high N rather than its role in nodulation. GA 20-oxidase 2 and 3 are among the enzymes regulating the flux of bioactive GA by catalyzing the final steps of GA biosynthesis (Gou et al., 2010). In *A. thaliana and tobacco* GA 20-oxidase is induced by auxin (Frigerio et al., 2006). The transport of auxin by AtPIN1 results in the degradation of

AUX/IAA proteins and activation of ARF7 TFs. This leads to the activation of GA biosynthesis genes, such as GA 20-oxidase (Saini et al., 2013).

The suppression of GA 20-oxidase 2 and 3 that results in the decrease in GA biosynthesis may affect the biosynthesis of ethylene. As studies show that low levels of GA can induce ethylene biosynthesis (Hayashi et al., 2014 and references within). The N-induction of ethylene-responsive transcription factor 5 (AC233556 27.1) that is involved in hormone metabolism at all time point in -Rhiz and +Rhiz may be related to this GA and ethylene interaction.

4.2.3.3.3 Regulation of protein activity

The overall expression pattern of the genes involved in protein metabolism (degradation and postranslational modification) showed some differences between different time points and/or -Rhiz and +Rhiz. For example, the degradation enzyme cysteine proteinase 2 was strongly N-induced at 2 hours of high N treatment and the posttranslational modification enzyme protein kinase-like protein was significantly N-induced at all time points in -Rhiz and +Rhiz. Carboxyl-terminal proteinase was strongly repressed at 6 hours in -Rhiz and at 2 hours in +Rhiz (Table 4-4). Around 70% of the differentially regulated genes involved in protein metabolism that responded to high N (in -Rhiz and +Rhiz) or rhizobia (at low N or 2 and 6 h high N treatments) were proteins mediating degradation (Tables 4-4 and 4-5). Protein degradation plays an important role in control of development and plant-microbe interaction (Hellmann and Estelle, 2002, El Yahyaoui et al., 2004) and is enhanced under stress or when the N or C of the amino acids is required for other processes (Vorster et al., 2013).

4.2.3.3.3.1 Rhizobial induction of putative players of nodule senescence in high N

Rhizobia had a strong (2.5 < FC < 7.5) effect on expression of nine differentially expressed cysteine proteinases (degradation enzymes), all of which were highly rhizobia-induced at low N and high N treatment (2 and 6 h), with the highest expression level at 6 h; except for Medtr3g044270.1 and

Medtr4g080730.1 that were significantly rhizobia-induced only in high N-treated plants (Table 4-5). The strongest rhizobia-induction of the cysteine proteinases was at 6 h post N-treatment, including Medtr5g022560.1 and AC233675 19.1 that based on MapMan pathway analysis have similarity to SENESCENCE-ASSOCIATED GENE 12 (SAG12) in *Arabidopsis* that is expressed in senescent tissues. Studies on nodule senescence in *Medicago* (Van de Velde et al., 2006) shows a high degree of overlap between the transcriptome of leaf senescence in *Arabidopsis* and nodule senescence in *Medicago*. This suggests that the two processes may recruit similar mechanisms. The induction of cysteine proteinases is a senescence marker in legumes as it is one of the main steps in the later stages of nodule development leading to senescence (Vorster et al., 2013). In *Glycine max*, as the nodule develops, the zone with highly expressed cysteine proteinases also increases in size (Alesandrini et al., 2003). During senescence metabolic and structural proteins are degraded and the assimilated N in the nodules is translocated from the nodule (Vorster et al., 2013).

The higher expression level of the cysteine proteinases (Table 4-5), especially the ones involved in senescence, in high N treated plants compared to plants grown at low N, could be an indication of nodule senescence. Since the nodules are no more than 2 weeks old and the lifespan of fast growing annual legumes is around 10-12 weeks (Vorster et al., 2013), it is more likely that this would be the very early stage of senescence possibly triggered prematurely by the N influx (high N treatment) that induces inhibition or reduction of nodule activities. Premature nodule senescence caused by stress such as nitrate treatment and dark stress (Matamoros et al., 1999) has been reported also in other studies. Studying the transcriptome of different stages of nodule senescence in *M. truncatula* has also identified a cluster of genes with enhanced gene expression before the initiation of nodule senescence (Van de Velde et al., 2006).

The two cysteine proteinases (Medtr5g022560.1 and AC233675 19.1) along with the sugar transporter Medtr6g006140.1 (solute carrier family 2

facilitated glucose transporter member 2) had the highest expression changes and were strongly rhizobia induced at 6 hours (Table 4-5). Based on MapMan analysis this sugar transporter is similar to the carbohydrate transmembrane transporter AtINT2 (INOSITOL TRANSPORTER 2). Medtr6g006140.1 shows high expression changes (FC>6) at low N and post treatment with high N at both time points. This gene shows to be affected by rhizobia more than the other significantly expressed genes (FC>8) at 6 hours pt with high N (having the highest FC among all the significantly expressed genes). This transporter is not well studied but it's strong rhizobia-induction at high N treated samples especially at the later time point (6 h) could be to retrieve carbohydrate compounds from the senescing nodules. As also studies on AtINT2 function in the leaf tissue suggests that it retrieves the diffused inositol from the mesophyll cells (Schneider et al., 2007).

The ammonium transporter 1 member 4 (Medtr7g098930.1) was induced by rhizobia and significantly expressed only at 6 h (FC>2). In another study looking at the nitrate impact on nodule activity in *M. truncatula*, Medtr7g098930.1 and some other members of the ammonium transporter family were expressed in the nodule (Cabeza et al., 2014). This transporter is highly similar (MapMan analysis) to the *Arabidopsis* AtAMT1;4 high affinity secondary active ammonium transmembrane transporter. The AMT transporter family contributes to the regulation of ammonium levels in the plants to prevent ammonium toxicity. AtAMT1;4 is one of the less characterized transporters in this family. It is a membrane localised transporter and it is believed to be involved in transporting ammonium into pollen. It contributes to N nutrition of the pollen by mediating the ammonium uptake and retrieval within the plasma membrane (Yuan et al., 2009). Here the over expression of this ammonium transporter at 6 h in response to rhizobia could be also related to ammonium retrieval as an early response to senescence.

4.2.3.4 Rhizobial and N co-regulation of development

A strong rhizobia effect upon gene expression was observed in plants grown at low N (0 time point) and also in seedlings treated with high N for (2 and 6 hour time points) in +Rhiz conditions (Figure 4-5 a). Remarkably, the genes significantly expressed under these conditions were highly upregulated (around 96%) (Table 4-1 and Figure 4-7 b). Cluster 26 (741 genes) from the study of clusters of differentially expressed genes (step 3 in Figure 4-2 c), was one of the clusters showing this strong rhizobia effect. In this cluster the N-effect on gene expression pattern was similar between -Rhiz and +Rhiz but the gene expression level was higher in +Rhiz (Figure 4-5 b). Around 47% of the genes in this cluster were significantly expressed genes involved in the nodulation pathway. The genes related to the nodulation pathway were mainly upregulated and showed very strong expression changes ($2 < FC < 9$) in response to rhizobia at low N (0 h) and high N (2 and 6 h).

The rhizobia-mediated induction of genes that were mainly involved in the nodulation pathway suggests an enhanced developmental activity in the roots under low and high N condition. The significant increase (FC>2) of the expression levels of differentially expressed genes at low N could be related to nodule organogenesis and activity. At high N the rhizobia-induction of genes (FC>2) may be associated to the rhizobia effect on the increased developmental activities when sufficient N is available. An example could be the rhizobia-induction of some enzymes involved in cell wall degradation during nodule organogenesis or LR emergence such as pectate lyases and polygalacturonases that had different expression levels at different time points (Table 4-5).

The nodulation pathway genes Medtr5g099060.1 and Medtr4g068000.1 that are *Nodule INception proteins* (*NIN*), a key regulator of nodulation (Marsh et al., 2007) and the differentially expressed calmodulin-like proteins 1, 2, 3, 4, 5, and 6b that are involved in calcium signaling were significantly induced ($2 < FC < 7$) at low and high N (2 and 6 h) with almost no variations in their

Table 4-6: Rhizobia-induced (FC>2 or FC<-2) nodulins at (a) low N and 2 h high N, (b) low N, (c) 2 h high N, and (d) 2 and 6 h high N treatment. Tables are sorted based on descending FC values of the first columns.

(a)

Nodulins (only expressed at Low N and 2 h high N)			
		FC	
		Low N (0 h)	High N (2 h)
AC146565_18.1	MtN11 protein	5.2	3.7
Medtr5g084080.1	Nodule specific glycine rich protein 1H	3.7	3.4
Medtr3g028380.1	NCR peptide 103	3.7	2.7
Medtr1g075500.1	NCR peptide 146	3.7	3.5
Medtr5g061060.1	NCR peptide 144	3.7	3.2
Medtr7g011480.1	NCR peptide 86	3.5	3.8
Medtr5g076040.1	NCR peptide 189	3.4	3.0
Medtr5g059440.1	NCR peptide 144	3.2	3.6
Medtr6g025330.1	NCR peptide 176	3.2	3.5
Medtr4g053600.1	NCR peptide 111	3.1	3.8
Medtr4g014790.1	NCR peptide 181	3.1	2.5
Medtr5g072310.1	NCR peptide 24	3.1	3.3
Medtr2g050060.1	NCR peptide 189	3.0	3.1
Medtr5g094210.1	MtN5 protein	3.0	2.0
Medtr5g084140.1	Nodule specific glycine rich protein 1E	2.9	2.6
Medtr7g071310.1	NCR peptide 328	2.9	2.5
Medtr4g053210.1	NCR peptide 111	2.8	3.4
Medtr7g114880.1	MtN26 protein	2.6	2.9
Medtr3g025420.1	NCR peptide 57	2.6	2.4
Medtr5g014080.1	NCR peptide 338	2.6	3.0
Medtr6g006360.1	NCR peptide 224	2.5	2.5
Medtr5g048310.1	NCR peptide 65	2.5	2.7
Medtr5g014050.1	NCR peptide 338	2.5	2.5
Medtr5g059670.1	NCR peptide 165	2.5	2.5
Medtr5g071880.1	NCR peptide 321	2.5	3.1
Medtr2g045290.1	NCR peptide 318	2.4	2.7
Medtr5g064860.1	NCR peptide 86	2.4	2.5
Medtr2g063470.1	NCR peptide 62	2.2	2.5
Medtr5g072280.1	NCR peptide 90	2.2	2.7
Medtr4g031900.1	NCR peptide 147	2.1	2.6
Medtr5g023780.1	NCR peptide 147	2.0	2.3

Table 4-6, continue

(b)

Nodulins (only expressed at Low N)		
		FC
Medtr5g069890.1	Nodule specific glycine rich protein 1B	3.6
AC146565_12.1	MtN11 protein	2.9
Medtr6g006350.1	NCR peptide 310	2.8
Medtr4g026680.1	NCR peptide 324	2.5
Medtr1g074860.1	NCR peptide 24	2.4
Medtr7g051290.1	NCR peptide 147	2.3
Medtr4g060720.1	NCR peptide 76	2.2
Medtr2g030480.1	MtN19 protein	2.0
Medtr5g047670.1	NCR peptide 339	2.0

(c)

Nodulins (only expressed at 2 h high N)		
		FC
Medtr1g043600.1	NCR peptide 57	3.0
Medtr7g080850.1	NCR peptide 62	2.7
Medtr5g066750.1	NCR peptide 24	2.5
Medtr5g032490.1	Nodule-specific glycine rich protein 1J	2.5
Medtr3g014720.1	NCR peptide 24	2.4

(d)

Nodulins (only expressed at high N)			
		FC	
		2 h	6 h
Medtr6g091440.1	NCR peptide 172	3.8	2.5
Medtr6g044570.1	Late nodulin	3.1	2.4
Medtr2g039230.1	Nodule specific glycine rich protein 6A	3.0	2.3
Medtr5g069540.1	NCR peptide 290	2.8	3.4
Medtr3g069890.1	NCR peptide 336	2.4	2.5

FC 2 5.2

expression levels between different time points and N concentrations (Table 4-5). Aside from calmodulin-like protein 3, all showed only rhizobia-regulated responses and did not show any strong N-regulated responses at 2 and 6 h of high N treatment in -Rhiz and +Rhiz. This may indicate that changes in the expression levels of these genes are in a rhizobia dependent manner and their role in regulation of nodulation is through a mechanism independent from that involving N availability.

There was not a strong N-regulation response among nodulins and rhizobia had a stronger effect on the expression of these genes. All differentially expressed nodulins were strongly ($2 < FC < 9$) rhizobia-induced at low and high N (2 and 6 h). The expression levels of these nodulins varied between N conditions and some showed significant expression changes only in certain N concentrations indicative of the level of nodule activity based on plant's N status (Table 4-6).

4.2.3.4.1 Nodule Specific Cysteine Rich (NCR) peptides

Around 80% of the nodulins showing rhizobia mediated induction at low and/or high N were members of the Nodule Specific Cysteine Rich Peptide (NCR) family. There were changes in the expression patterns of different sets of NCRs depending on the N status of the seedlings (growth at low N, treatment with high N for 2 and 6 h) (Table 4-5 and 4-6). NCRs have around 500 members in *Medicago* with unclear biological function (Marshall et al., 2011, Silverstein, 2014). They are a class of cysteine rich peptides (CRP) and are only found in *Medicago* and some other closely related legume families (Farkas et al., 2014). NCRs suppress the rhizobial reproduction and increase the ploidy levels hence causing the irreversible differentiation of the rhizobia in the symbiosomes into bacteroids with the ability to fix N (Van de Velde et al., 2010). NCRs are also one of the components of the plant's defence reaction to bacteria that turns infection into the beneficial rhizobia-legume symbiosis interaction (Farkas et al., 2014). There are evidences for the involvement of CRPs in different molecular signaling pathways and some NCRs are also

candidates for regulating nodule development (Wang et al., 2010, Van de Velde et al., 2010, Marshall et al., 2011). Here we have identified 6 differentially expressed NCRs (Table 4-6 b) that are significantly (FC>2) upregulated in response to rhizobia at low N. These NCRs could be involved in the nodule development pathway because of their enhanced expression levels at low N where to compensate for the N deficiency under N deprivation plants showed increased nodulation activities (as observed at the our phenotyping studies).

We have also identified NCRs with putative role in control of nodule number or inhibition of nodule activity at high N (Table 4-6 c and d) because of their exclusive strong induction (FC>2) over high N treatment at 2 and/or 6 h. It is also interesting to know whether these NCRs also affect LR development at high N and in the presence of rhizobia. The over expression of these NCRs only at high N could make them one of the putative players in the rhizobia effect on LR length (Figure 4-1) as observed in our phenotypic studies. Several studies have provided evidences that CPRs may function as secreted signaling peptides regulating several aspects of development and secreted CRPs are identified in the roots having essential roles in root development (Marshall et al., 2011) and references within.

4.3 Gene expression responses to low treatment in rhizobia inoculated seedlings grown on high N

A microarray experiment was designed (Figure 4-2 b) to study the response of rhizobia inoculated seedlings grown at high N to a 6-hour treatment with low N. This was to study the effect of transferring from high N (5 mM) condition to low N condition (0.1 mM) on the gene expression. Data was quality checked and normalised as carried out earlier (section 4.1).

The Limma package in R (Smyth, 2004) was used to identify 2 patterns of significantly different gene expression for 2 treatments (HH, HL) with a significance cutoff of $P<0.05$. From a total of 47529 genes tested on the Nimblegen Mt3.5 gene expression array, 168 differential expressed genes

Table 4-7: Rhizobia mediated N-regulation of genes with significant gene expression changes (FC>2 or FC<-2) in response to low N treatment of rhizobia inoculated samples grown at high N assigned to different functional categories using MapMan software (Thimm et al., 2004). Tables are sorted alphabetically by the functional categories column.

Low N-regulated responses				
	Functional category	**Gene ID**	**Annotation**	**FC**
N metabolism	nitrate metabolism	Medtr3g073180.1	Nitrate reductase	-2
Transporter	nitrate transporter	Medtr2g085510.1	High-affinity nitrate transporter	2
secondary metabolism	flavonoids.dihydroflavonols.dihydroflavonol 4-reductase	Medtr7g074870.1	Dihydroflavonol 4-reductase	-2
	flavonoids.dihydroflavonols	Medtr7g074880.1	Dihydroflavonol-4-reductase	-3
Hormone metabolism	abscisic acid synthesis-degradation	Medtr7g045370.1	Carotenoid cleavage dioxygenase 7, chloroplastic	2
	auxin induced-regulated-responsive-activated	Medtr7g114980.2	Aldo/keto-reductase family protein	-2
Nodulation		Medtr5g084260.1	Nodule-specific glycine-rich protein 1L	-2
Transcriptional regulators	MYB-related transcription factor family	Medtr5g020540.1	MYB transcription factor MYB164	2
	bZIP transcription factor family	AC233556_19.1	Transcription factor bZIP	2
	bHLH,Basic Helix-Loop-Helix family	Medtr3g116770.1	Transcription factor BEE 2	-2
	G2-like transcription factor family, GARP	Medtr5g017980.1	Two-component response regulator ARR1	-2

FC -3 2

were identified. Two clusters were formed of these differentially expressed genes. Low N was found to repress 73% of the differentially expressed genes (cluster 2 with 123 genes).

The effect of low N treatment after growth at high N condition on the gene expression was studied by calculating the FC between high N treated (control) and low N treated samples. To analyse the regulation of gene expression at higher stringency, differentially expressed genes from Limma analysis that had a FC>2 or FC<-2 were determined as significant. Only 21% of the differentially expressed genes were significantly expressed of which 83% were downregulated (Table 4-7). This predominant gene suppression in response to low N treatment could suggest the activation of a local N signal for decreasing biological activities at limiting N condition. NRT2.1 may be acting as a nitrate sensor and signal transducer in this response because of the strong N-induction (FC>2) of the differentially expressed high affinity nitrate transporter Medtr2g085510.1 (NRT2.1). Consistent with previous findings in *Arabidopsis* (Remans et al., 2006b, Little et al., 2005), here NRT2.1 could be also involved in regulating LR development and suppression of LR initiation at low nitrate. The strong N-suppression (FC<-2) of nitrate reductase Medtr3g073180.1 could be another indicative of the decrease in N assimilation activities in response to low N.

MYB164 (Medtr5g020540.1), bZIP (AC233556_19.1), BEE2 (Medtr3g116770.1) and two-component response regulator ARR1 (Medtr5g017980.1) were the only differentially expressed TFs showing significant (FC>2 or FC<-2) expression changes under low N treatment. ARR1 (from the G2-like TF family) and BEE2 (from the bHLH TF family) were strongly N-repressed (FC<-2) while MYB164 and bZIP TFs showed strong N-induction (FC>2). These TFs may be involved in the regulation of the rhizobia mediated responses to low N (Table 4-7) affecting N assimilation and transport because of their co-expression with NRT2.1 and nitrate reductase. Network analysis has also identified putative roles for AtbZIP and AtMyb TFs in controlling nitrate responses in *Arabidopsis* (Canales et al., 2014). This

study has also provided evidence for the role of G2-like TFs in regulating transport and signaling functions in response to nitrate.

LR formation is promoted through the synergic function of BRs and auxin (Bao et al., 2004). BR are also negative regulators of nodule numbers possibly through unknown shoot-derived AON signaling pathways (Ryu et al., 2012). In *Arabidopsis*, BR facilitates polar auxin transport by enhancing AtPIN expression. Polar auxin transport and local accumulation are essential for LR formation and nodule organogenesis. Thus the level of BR and auxin could affect LR development and nodulation (Ryu et al., 2012). The co-expression of BEE2 (BRASSINOSTEROID ENHANCED EXPRESSION2) TF that is involved in early BR responses (Friedrichsen et al., 2002) along with the aldo/keto-reductase family protein Medtr7g114980.2 (Table 4-7) that is involved in auxin metabolism may be indicative of the roles they have in the regulatory cross talk between BR and auxin in regulating LR development and nodule number. BEE2 along with BEE1 and BEE3 have a significant role in plant growth and they redundantly promote cell elongation (Friedrichsen et al., 2002). Studies in *Arabidopsis* have showed that BEE2 expression is repressed by ABA, an antagonistic of the BR pathway (Friedrichsen et al., 2002) and this crosstalk between ABA and BR pathway is important in regulating plant development. The strong N-suppression of BEE2 (FC<-2) along with the N-induction of the carotenoid cleavage dioxygenase 7 (Medtr7g045370.1) that is involved in ABA metabolism may suggest that these two genes are involved in the BR and ABA pathways to regulate developmental activities in the root in response to low N.

4.4 Study of gene expression changes at cell type level

Plant responses to the environment occur at a cell-specific level (Spaink, 2000, Gifford et al., 2008). Nodules and LRs are developed from cortex and pericycle respectively. Thus the aim of this research was to study the gene expression changes in response to N concentration and rhizobia at cortex and pericycle. This could be achieved by producing transgenic lines expressing GFP in pericycle or cortex. Fluorescently-Activated Cell Sorting (FACS)

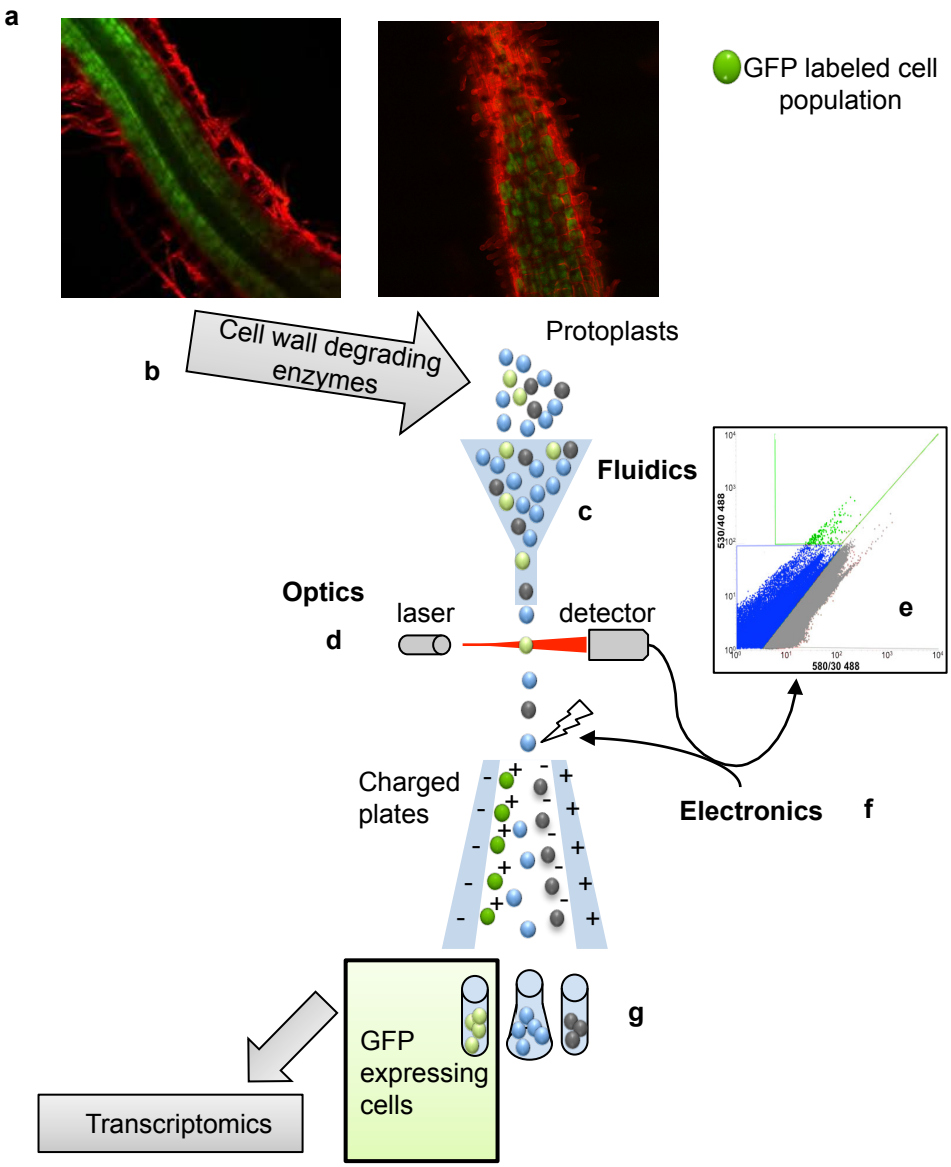


Figure 4-9: Fluorescence-Activated Cell Sorting (FACS) workflow. (a) GFP expression in transgenic *M. truncatula* A17 cortex (left) and pericycle (right) cells (Gifford, unpublished results); (b) treatment and filter of the harvested roots with enzymes to dissociate cells; (c) sheath fluid sort stream containing the cell sample that is vibrated at high frequency to break into uniform droplets containing one cell each; (d) laser light and detection filters that measure the fluorescence and other properties (such as size) of each droplet; (e) analysis of the emission spectrum; (f) electrical charge imparted on droplets; (g) electrical plates deflect charged droplets, here illustrated for two-way sorting (green/grey shaded cells) with a waste collection of all other cells (blue).

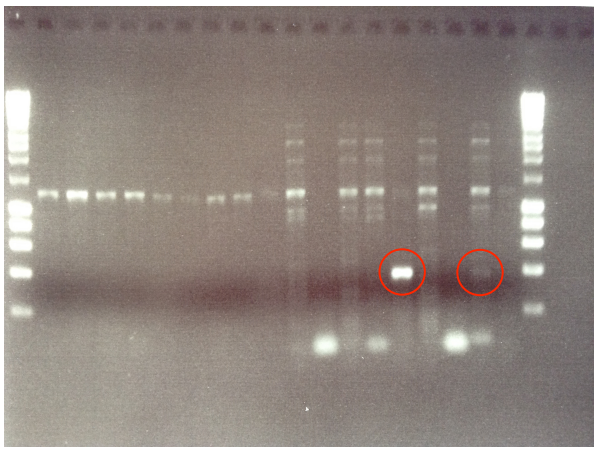


Figure 4-10: GFP expression in the successful transformed plants was identified using PCR. Bands 15 and 18 (red circles) are showing GFP presence. From left to right: Bands 2-10= control, 11-19= GFP.

(Carter et al., 2013) could be used to isolate single cell types of interest (Figure 4-9). Gene expression changes of the root cortex or pericycle samples could be then studied using microarrays.

M. truncatula var. Jemalong A17 were transformed to produce GFP-marked pericycle or cortical cell type plant lines (Figure 4-9 a) using transformation methods explained in section 2.7 (Zhou et al., 2004). The lines were tested for the insertion of GFP by PCR (Figure 4-10).

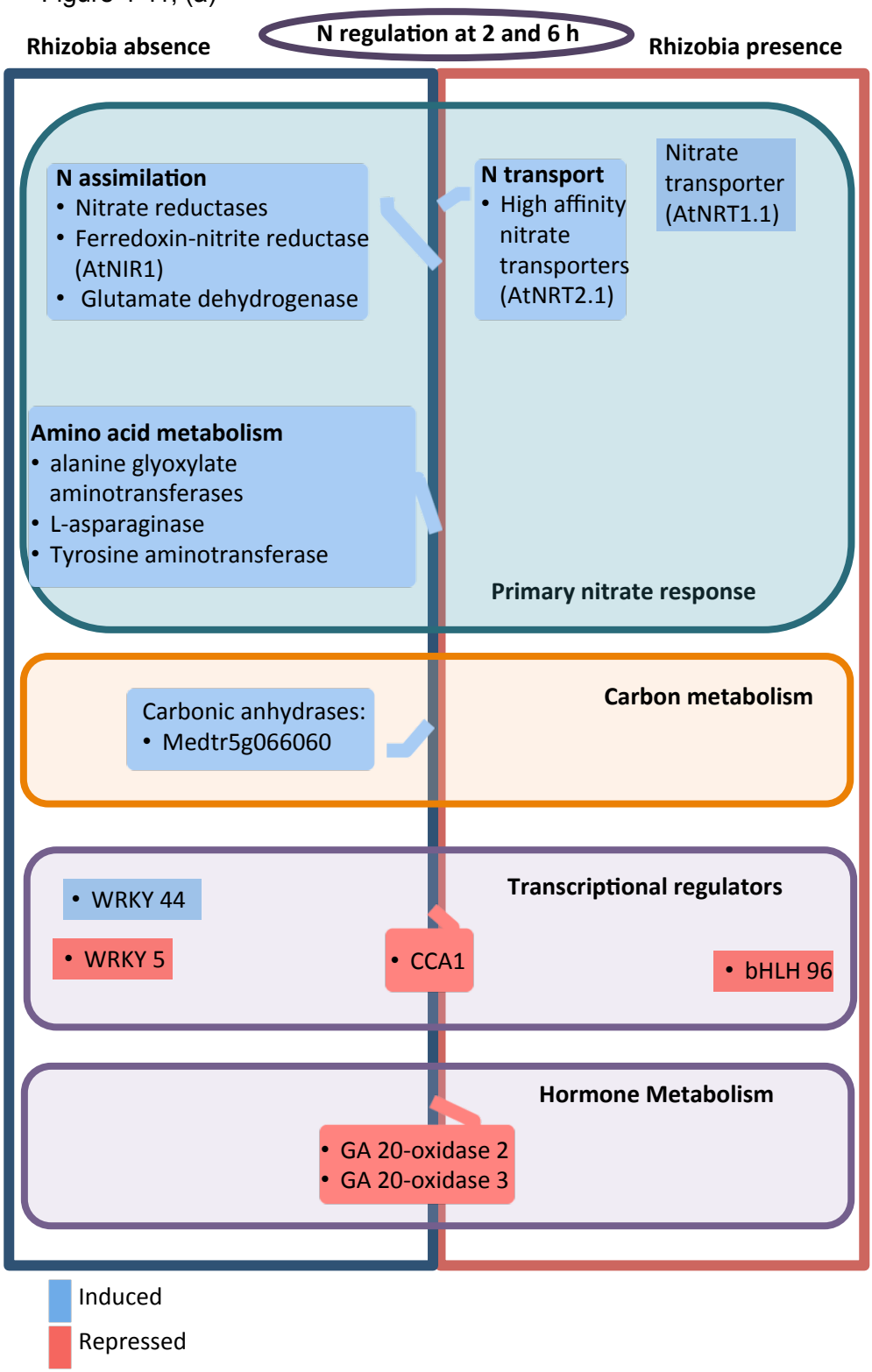
In order to have sufficient RNA (~80ng) for running microarray around 2,000 plant cells need to be isolated by FACS. Although the GFP expression in early generations (T1 and T2) was strong and cell type specific but it was silenced in later generations (T3 and T4) so they could not be used for transcriptomic studies. Hence, it was not possible to continue the following studies at cell-specific level.

4.5 Conclusion

We have identified a total of 4793 genes that were differentially expressed at 0, 2 and 6 h post treatment with high N in -Rhiz and +Rhiz samples previously grown at low N for 14 days. This represented 7.4% of the genes annotated in the *M. truncatula* 3.5 gene model. The significantly expressed genes with FC>2 or FC<-2 were assigned to biological functional categories using MapMan to identify the main biological pathways controlled by N or rhizobia mediated N signals (Figure 4-7). Transport, metabolism and transcriptional regulation of root responses to N were among the biological functional categories showing the strongest responses to N (Figure 4-7 a) as this is also consistent with the data obtained in *Arabidopsis* (Canales et al., 2014). The rhizobia mediated responses to N were mainly observed in transport, signaling, regulation of protein activity, and development functional categories (Figure 4-7 b).

A model was generated for the biological functions regulated by N in the absence or presence of rhizobia (Figure 4-11 a) and also rhizobia mediated N

Figure 4-11, (a)


Rhizobia absence
N regulation at 2 and 6 h
Rhizobia presence
N assimilation
• Nitrate reductases
• Ferredoxin-nitrite reductase (AtNIR1)
• Glutamate dehydrogenase
N transport
• High affinity nitrate transporters (AtNRT2.1)
Nitrate transporter (AtNRT1.1)
Amino acid metabolism
• alanine glyoxylate aminotransferases
• L-asparaginase
• Tyrosine aminotransferase
Primary nitrate response
Carbonic anhydrases:
• Medtr5g066060
Carbon metabolism
• WRKY 44
• WRKY 5
Transcriptional regulators
• CCA1
• bHLH 96
Hormone Metabolism
• GA 20-oxidase 2
• GA 20-oxidase 3
Induced
Repressed

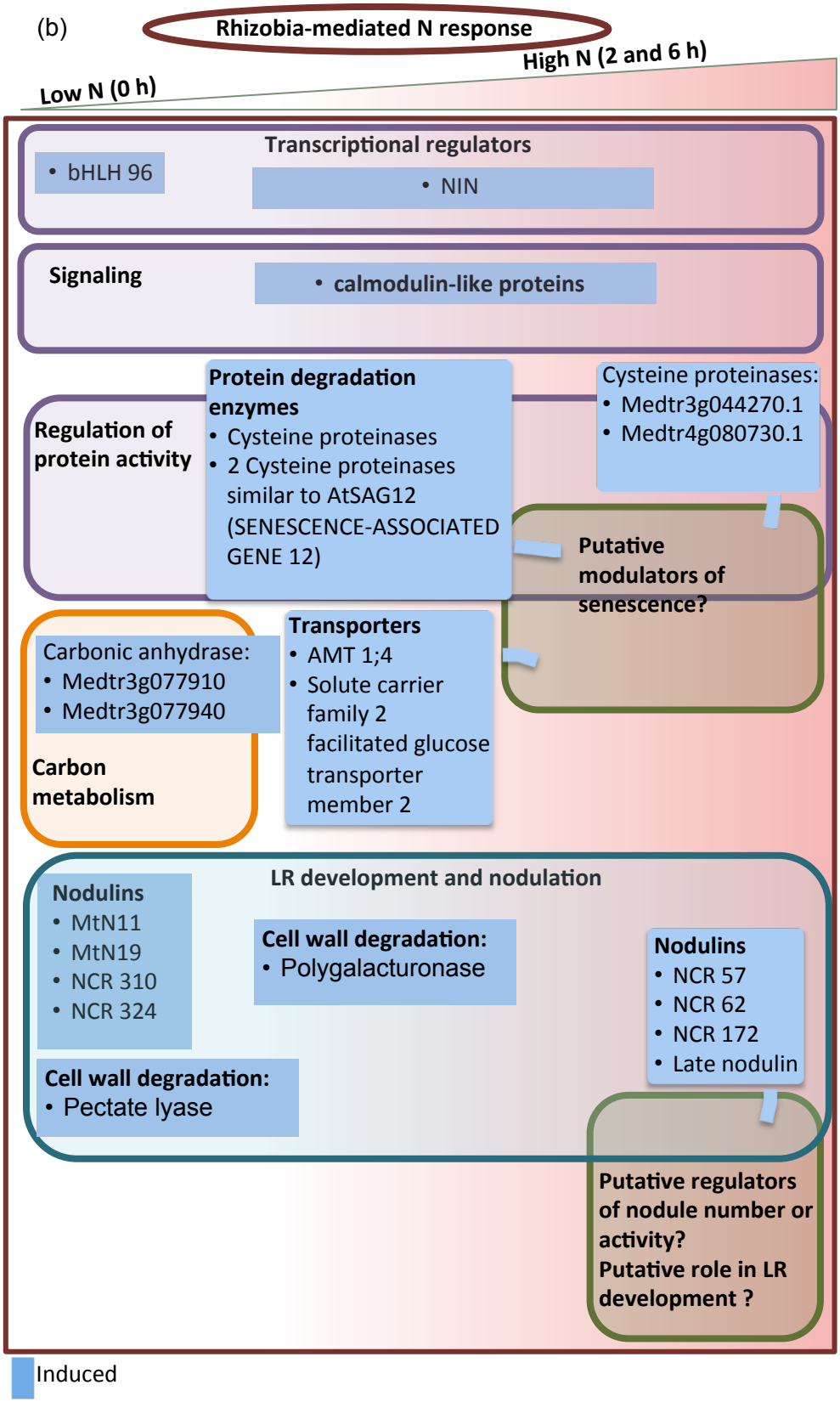


Figure 4-11: Candidate upregulated (blue) and down regulated (red) genes potentially involved in (a) N-regulated and (b) rhizobia-mediated N responses. These are genes that showed a significant response (FC>2 or FC<-2) to N at 2 or 6 h after high N treatment in mock-inoculated (-Rhiz) or rhizobia-inoculated (+Rhiz) in (a) or were significantly regulated (FC>2 or FC<-2) by rhizobia at low (0 h) or high (2 and 6 h) N in (b). In (a) the boxes pointing to the two red and opaque lines in the middle indicate that the genes are expressed in both -Rhiz and +Rhiz.

responses at low or high N in A17 (Figure 4-11 b). The early (2 and 6 h) N responses in N-deprived plants provided with high N resulted in the induction of primary nitrate response genes (Figure 4-11 a). The two nitrate sensor and transporters NRT2.1 and NRT1.1 were one of the key players in this response. The strong induction (FC=2.5) of NRT1.1 only in the presence of rhizobia could be a regulatory response affecting the root phenotype at high N in rhizobia-inoculated plants. It seems that the non-nodulating plants (-Rhiz) grown under low N, showed a more enhanced primary nitrate response (e.g. induction of genes involved in N transport and assimilation, and amino acid metabolism) than the nodulating (+Rhiz) samples. Thus, this enhanced activity could be to compensate for the N deficiency they were facing. The stronger induction of ferredoxin-nitrite reductase (Table 4-4) involved in N assimilation and the higher number of amino acid metabolism genes in -Rhiz could be an indication of this enhanced response. Distinct sets of TFs were involved in the regulation of N responses in high N (2 and 6 h) treated rhizobia inoculated and mock inoculated plants (Figure 4-8 c and Figure 4-11 a) and also rhizobia-regulated responses at low and high N (Figure 4-8 d and Figure 4-11 b). Some of the TFs were however similar between different conditions with variations in their expression. The strong N-suppression of the circadian clock-associated protein 1a (Medtr8g077420.1) that was related to the duration of high N treatment and independent of rhizobia confirms the results from other studies that this TF could be one of the regulators of the N assimilation (Gutiérrez et al., 2008, McClung, 2006). The N-responsive expression changes of WRKY44 (FC>2) and WRKY5 (FC<-2) TFs exclusively in the absence of rhizobia may be indicative of their involvement in N responses in N-deprived plants when N is available.

Significant expression changes in some of the genes involved in different pathways related to development and regulation (TFs, hormonal regulation and signaling) indicates enhanced developmental activities early (2 and 6 h) after treatment with high N. Strong induction (FC>2) of some of the TCA cycle enzymes and proteins involved in carbohydrate metabolism specifically TCA carbonic anhydrases (Medtr5g066060) in both non-

nodulating and nodulating plants could be to provide the necessary carbon and energy pool for the enhanced metabolic and developmental activities due to N availability (Figure 4-11). The rhizobia induction and enhanced expression of two cysteine proteinases (Medtr5g022560.1 and AC233675 19.1) that are known as senescence markers and two sugar and ammonium transporters (Medtr6g006140.1 and Medtr7g098930.1 respectively) in 6 h high N treated plants may be indicative of early stages of a premature senescence to inhibit nodulation in order to use the carbon and energy sources for other developmental activities when enough N is available (Figure 4-11).

Changes in the RSA are under the control of N availability. N is also one of the key factors controlling the balance between the number and activity of nodules and LR development. Here we have identified some of the components of this regulatory complex (Figure 4-11). The two TCA carbonic anhydrases Medtr3g077910 and Medtr3g077940 could be involved in regulating the number of nodules because of their strong ($3 < FC < 7$) rhizobia induction at low N and not at high N (2 and 6 h). The expression level of these enzymes could be related to the level of nodule development and functioning in N-deprived plants. They may function to provide the necessary photoassimilates for the nodule activity and provide carbohydrates to the bacteroids in the active nodules. The rhizobia-dependent expression of bHLH96 TF (strong rhizobia induction at low N and strong N-suppression in +Rhiz) could suggest that this TF may be involved in the regulation of nodulation or LR development. The strong rhizobia-induction ($FC>2$) of 6 NCR peptides only at low N could make them some key players in the nodulation pathway (Table 4-6 b). A small group of NCRs also showed strong rhizobia-induction ($FC>2$) specifically at high N (Table 4-6 c and d) making them putative regulators/inhibitors of nodule development at high N. These NCRs could be also candidates for the rhizobia-mediated LR development observed at high N condition (Figure 4-1). The strong N-suppression ($FC<-3$) of GA 20-oxidase 2 and 3 that was independent of the presence or absence

of rhizobia and was related to the duration of high N treatment may be related to their regulatory role in LR development at high N condition.

A model was also proposed for the rhizobia mediated response to low N in the rhizobia-inoculated plants grown under high N condition (Figure 4-12). The predominant (83%) suppression of the genes with significant expressed changes (FC>2 or FC<-2) suggests the activation of a local N signal for decreasing biological activities at low N condition. NRT2.1 that is strongly induced by low N (FC>2) could be acting as a signal molecule in regulating this early response to low N and the strong suppression (FC<-2) of nitrate reductase also suggests the decrease in N assimilation at low N. The significant expression of BEE2 TF and two enzymes involved in auxin and ABA metabolism may be indicative of the hormonal cross talk between BR and auxin and also between BR and ABA in controlling the number of nodules and LR development. ABA regulates the expression of BEE2 TF that is one of the regulators of BR response. BR is involved in controlling the number of nodules and LR development by the positive effect it has on polar auxin transport and accumulation.

The significantly expressed genes (FC>2 and FC<-2) identified by studying the gene expression changes in response to high N (0, 2 and 6 h time points) and rhizobia (Figure 4-11) could be involved in regulating root development and nodulation at high N (Figure 4-1). Some of the components of AON for controlling the number of nodules are also involved in regulating LR development. Thus, gene expression changes in response to N (low and high) and rhizobia were compared between A17 and *sunn-1* mutant. This mutant is impaired in the correct AON signals that lead to hypernodulating phenotype with short LRs at low N. This study aimed to identify (1) genes showing N and/or rhizobia regulated responses (these could be putative AON regulators also involved in the balance between LR and nodule development), (2) genes showing significant *SUNN*-mediated expression changes in response to N and rhizobia, (3) biological pathways involved in the cross talk

between AON and LR development. In chapter 5 these studies will be discussed in detail.

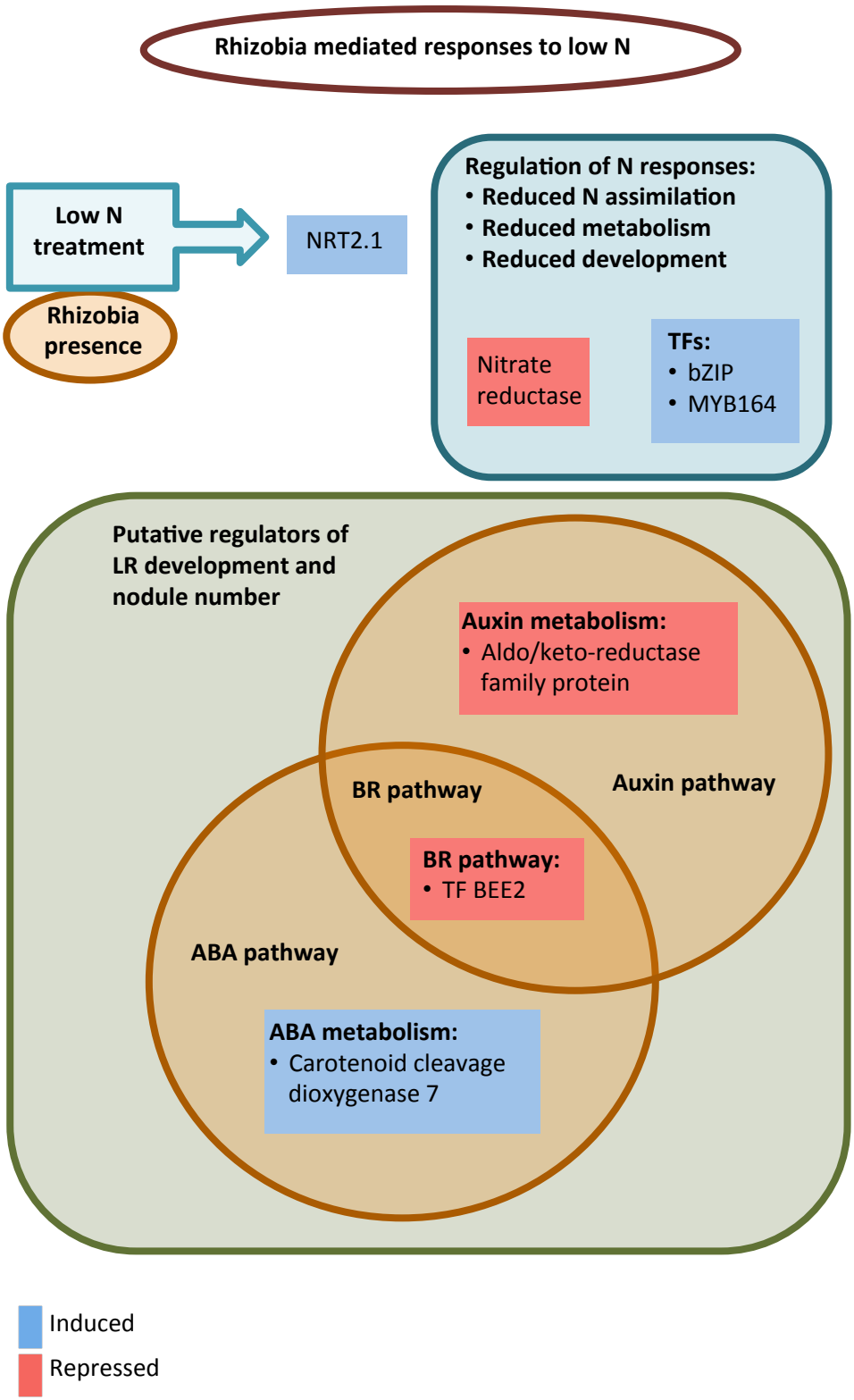


Figure 4-12: Candidate upregulated (blue) and downregulated (red) genes potentially involved in rhizobia mediated responses to low N. These are genes that showed a significant response (FC>2 or FC<-2) to N 6 h after low N (0.1) treatment of rhizobia inoculated plants grown at high N (5 mM).

Chapter 5

Results: Analysis of hypernodulation mutant *sunn-1* implicates balancing genes to regulate LR development

Analysis of *M. truncatula* A17 (chapter 3 and 4; (Bonyadi Pour et al., 2014 In Prep) in this study and other comparable research (Mathesius, 2003) suggest that factors such as rhizobia and N level can affect both nodulation and LR development. This effect could be at the level of cross talk between signaling and developmental pathways, possibly mediated by genetic or protein-protein interactions. For example, high levels of N in the soil or in the plant inhibits the formation of nodules via AON mechanisms, controlled by *super numeric nodules (MtSUNN*) in *M. truncatula* A17 (Schnabel et al., 2005), and *Hypernodulation Aberrant Root formation (LjHAR1*) in *L. japonicus* (Wopereis et al., 2000). This thesis has shown that in A17 the presence of rhizobia at high levels of N that inhibit nodulation also represses PR and LR development (chapter 3 and Figure 4-1). This suggests tight coregulation of LR and nodule formation, with connection of gene regulatory pathways a likely mechanism underlying this. There are studies suggesting that AON genes could be candidates for regulating LR development in response to N (Jin et al., 2012). A17 and the hypernodulating mutant *sunn-1* that is impaired in correct AON responses (Van Noorden et al., 2006) exhibit different responses to low and high N concentrations and rhizobia inoculation (Jin et al., 2012). This mutant has a defect in regulating the number of root organs due to increased shoot to root auxin transport compared to the wild-type (Van

Noorden et al., 2006). We have utilized the *sunn-1* mutant because of its hypernodulating phenotype and short LRs at low N and the ability to nodulate at high N to compare the responses of this mutant with A17 at low and high N and in response to rhizobia. This could give us an understanding of how *SUNN* could be involved in changing root architecture to balance LR development with nodule formation according to the plant N supply.

5.1 Characterizing *SUNN* mediated changes on root architecture in response to N and rhizobia

To compare *sunn-1* root architecture changes to low and high N and rhizobia inoculation with A17, -Rhiz and +Rhiz plants were grown at low (0.1 mM) and high (5 mM) N and root attributes (Figure 2-2) of 2 biological replicates (n=6 plants per replicate) were measured at 14 dpi (Figure 3-1). Square root transformation was applied on the data and REML (P<0.05) was used to study the effect of genotype, N concentration, rhizobia inoculation and the interaction of between these main effects on root development and nodulation (Table 5-1).

The study shows that at low N the number of nodules was significantly (P<0.05) higher in *sunn-1* compared to A17 (Figure 5-1 a) and it was affected (P<0.05) by genotype and N concentration (Table 5-1). Genotype, N concentration and rhizobia also had significant (P<0.05) effect on root development (Table 5-1). There was a significant (P<0.05) genotype effect on PR length, average of LR length and LR number. The effect of N concentration was significant (P<0.05) on LR development (LR total length and LR number). Rhizobia effect on root development was also significant (P<0.05) on PR length, average of LR length, LR total length, LR number and root total size (Table 5-1).

The mean values for different root attributes in +Rhiz and -Rhiz samples at low and high N were compared between A17 and *sunn-1* using a two sample Student's t-test at 5% significance level. The results showed that the difference between A17 and *sunn-1* root phenotype was significant (P<0.05)

Table 5-1: *P* values from (a) REML and (b) t-test analysis of the differences between trait values in rhizobia inoculated and mock inoculated A17 and *sunn-1* samples at low and high concentrations of N. PR= primary root, LR= lateral root.

(a)

Effect of low and high N concentrations and rhizobia on root system architecture of A17 and *sunn-1*

	F pr						
	PR length	**Average of LR length**	**LR total length**	**LR density**	**Total root size**	**LR number**	**Nodule number**
Genotype	0.013*	<0.001*	0.44	0.013*	0.144	<0.001*	0.015*
N concentration	0.083	0.78	0.034*	<0.001*	0.199	0.003*	<0.001*
Rhizobia	<0.001*	0.031*	<0.001*	0.673	<0.001*	0.031*	
Genotype x N concentration	<0.001*	0.711	0.158	0.763	0.031*	0.038*	0.258
Genotype x rhizobial	0.717	0.004*	0.358	0.139	0.61	0.379	
N concentration x rhizobia	0.098	0.183	0.479	0.414	0.216	0.063	
Genotype x N concentration x rhizobia	0.473	0.084	0.112	0.626	0.284	0.742	

* $P < 0.05$ in REML variance components analysis

(b)

Student t-test comparison between A17 and *sunn-1*

	N concentration (mM)	**PR length**	**Ave LR length**	**LR total length**	**LR density**	**Total root size**	**LR number**	**Nodule number**
-Rhiz	**0.1**	0.167	0.672	0.171	0.200	0.468	0.353	
	5	0.392	0.878	0.361	0.018*	0.586	0.175	
+Rhiz	**0.1**	0.868	0.001**	0.055*	0.089	0.115	0.325	0.053*
	5	0.002**	0.004**	0.043*	0.008**	0.014*	0.0002**	

* = $0.01<P<0.05$ and ** = $P<0.01$

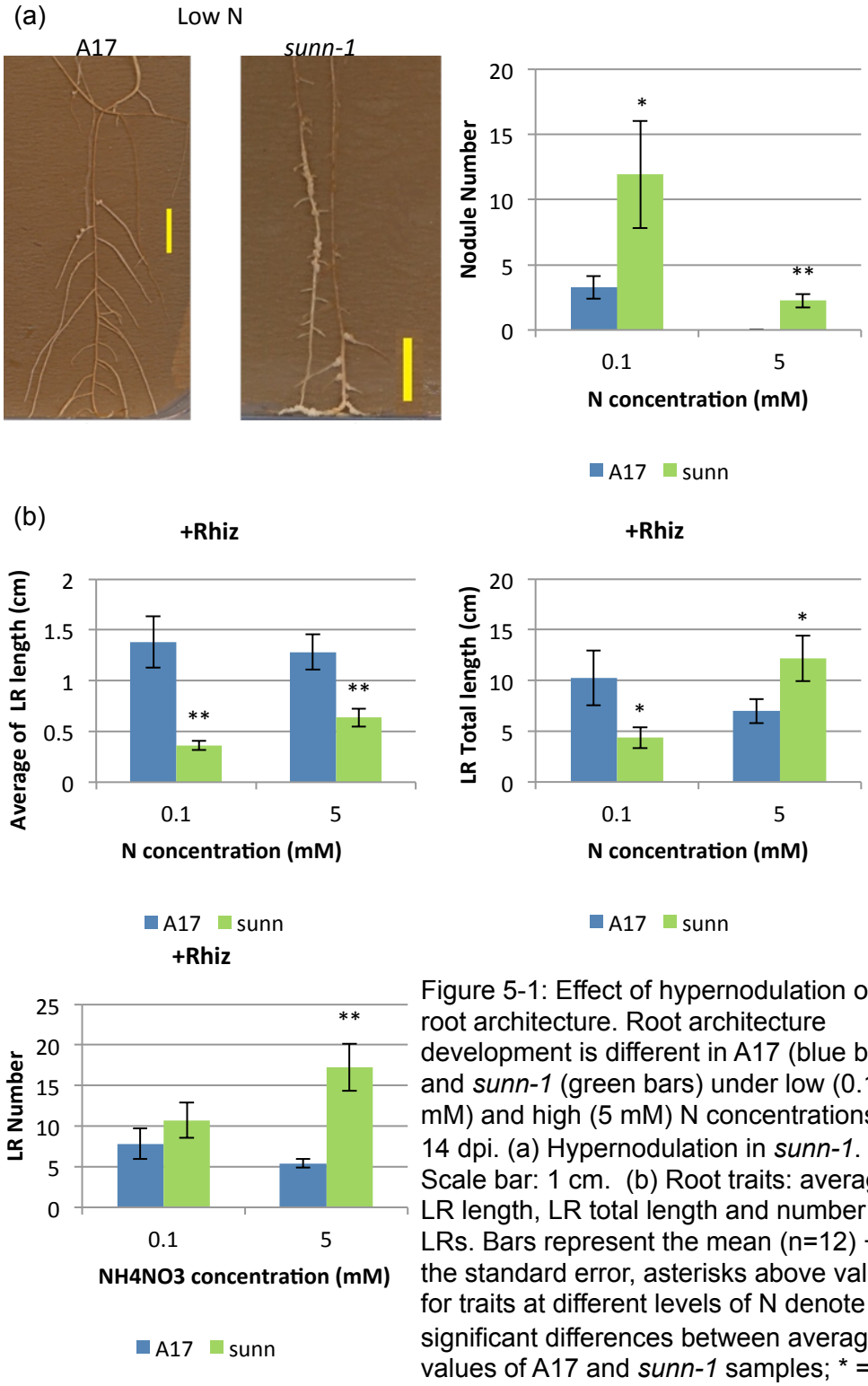


Figure 5-1: Effect of hypernodulation on root architecture. Root architecture development is different in A17 (blue bars) and *sunn-1* (green bars) under low (0.1 mM) and high (5 mM) N concentrations at 14 dpi. (a) Hypernodulation in *sunn-1*. Scale bar: 1 cm. (b) Root traits: average of LR length, LR total length and number of LRs. Bars represent the mean (n=12) +/- the standard error, asterisks above values for traits at different levels of N denote significant differences between average values of A17 and *sunn-1* samples; * = 0.01 < P <0.05 and ** = P <0.01 in t-tests.

between +Rhiz samples (as also confirmed by REML showing significant (P<0.05) rhizobia effect on root development and nodule number).

In A17, the rhizobia effect on root development was not significant at low N (chapter 3). At high N rhizobia significantly (P<0.05) affected root development in A17. The total root size was significantly (P<0.01) less in +Rhiz compared to -Rhiz and this was due to significantly (P<0.01) shorter PR, smaller LR total length and less LR in number. The response of *sunn-1* to low and high N and rhizobia inoculation was different from A17. At low N, LRs were shorter in nodulating *sunn-1* samples compared to A17, which was specific to nodulating plants, not in -Rhiz samples. Average of LR length was significantly (P<0.01) less in +Rhiz *sunn-1* compared to A17 at low N. This was because of a smaller (P<0.05) LR total length and not due to the number of LRs (Figure 5-1 b). There was not a significant (P<0.05) difference in LR number (Figure 5-1 b) and PR length (Table 5-1 b) at low N between *sunn-1* and A17.

At high N that nodulation is inhibited in A17, *sunn-1* had the ability to nodulated but the number of nodules were significantly (P<0.05) less in high N compared to low (Figure 5-1 a). Total root size was significantly (P<0.05) higher in nodulating *sunn-1* samples compared to A17 because of a significantly (P<0.01) longer PR and higher (P<0.05) LR total length. The average of LR length was significantly (P<0.01) less in +Rhiz *sunn-1* compared to A17 at high N suggesting that higher total LR length in *sunn-1* was due to a higher number of LRs (average of LR length is less in *sunn-1*).

The shorter LRs and hypernodulation in the presence of rhizobia at low N in *sunn-1* compared to A17 and longer PR, higher LR total length (smaller average of LR length) and higher number of LRs at high N could suggest a balancing effect for *SUNN* on nodulation and root development specifically in the presence of rhizobia as also reported in a study looking at the involvement of *SUNN* in root architecture responses to N (Jin et al., 2012).

The N content in root and shoot of +Rhiz and -Rhiz samples of A17 and *sunn-1* at low and high N was measured by estimating the free nitrate and total N. A17 and *sunn-1* +Rhiz and -Rhiz plants in 3 biological replicates (n=54) were grown under low and high (Figure 3-1) and at 14 dpi wet and dry weight of root and shoots measured. The 3 replicates were pooled together to provide enough plant material for the N measurements. Free nitrate was measured in root and shoot and enough samples were available to measure total N only in the shoot. The data obtained from these measurements could not be used for studying N acquisition efficiency (Moreau et al., 2012) in A17 and *sunn-1* due to lack of enough biological replicates.

REML (P<0.05) was applied on the Log_{10} transformed root dry weight (representing the root surface area) and shoot dry weight to study the effect of genotype, N concentration, rhizobia inoculation and the interaction between these main effects (Table 5-2 and figure 5-2). This analysis showed that there was a significant (P<0.05) genotype effect on shoot and root dry weight. Shoot dry weight was also significantly (P<0.05) affected by N concentration and the interaction between N concentration and rhizobia. Effects of rhizobia, N concentration and the interaction between the three main factors were significant (P<0.05) on root dry weight (Table 5-2). The comparison of mean values for root and shoot dry weight between A17 and *sunn-1* using Student t-test (P<0.05) showed that there was no significant difference in the root area between A17 and *sunn-1* at low or high N in -Rhiz or +Rhiz samples (Figure 5-2). Thus, irrespective of significantly (P<0.05) higher number of nodules in *sunn-1* compared to A17 (at low and high N) there is not a significant (P<0.05) difference between their root areas. This shows that there is tight control on the allocation of carbon resources for LR and nodule development in the root and the hypernodulation in *sunn-1* is balanced with inhibition of LR elongation (Figure 5-1).

Concentration of shoot and root free nitrate and shoot total N in A17 and *sunn-1* samples were similar at low N (Figure 5-3). This could suggest that hypernodulation at low N had no effect on the N content of root or shoot. This

Table 5-2: REML *P* values from analysis of the differences between shoot and root dry weight of A17 and *sunn-1* rhizobia or mock inoculated samples at low and high concentrations of N.

	F pr	
	Shoot dry weight	**Root dry weight**
Genotype	<0.001**	0.005**
N concentration	0.002**	0.422
Rhizobia	0.113	0.017*
Genotype x N concentration	0.459	0.019*
Genotype x rhizobia	0.102	0.286
N concentration x rhizobia	0.034*	0.081
Genotype x N concentration x rhizobia	0.192	0.010**

* *P* < 0.05 in REML variance components analysis

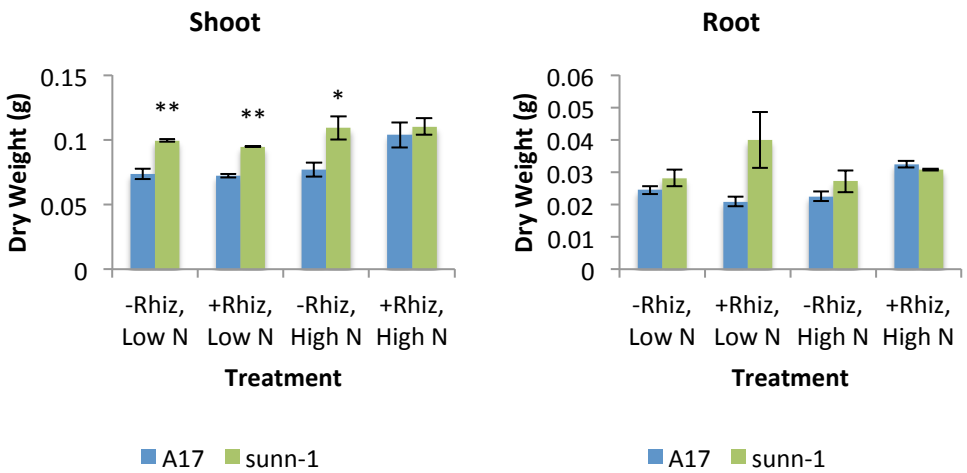


Figure 5-2: Comparison of shoot and root dry weight between A17 (blue bars) and *sunn-1* (green bars) rhizobia inoculated (+Rhiz) or mock inoculated (-Rhiz) samples at low (0.1 mM) and high (5 mM) concentrations of N. Bars represent the mean (3 biological replicates, n=18 plant per replicate) +/- the standard error, asterisks above values for traits at different levels of N denote significant differences between average values of A17 and *sunn-1* samples; * = 0.01 < P <0.05 and ** = P <0.01 in t-tests.

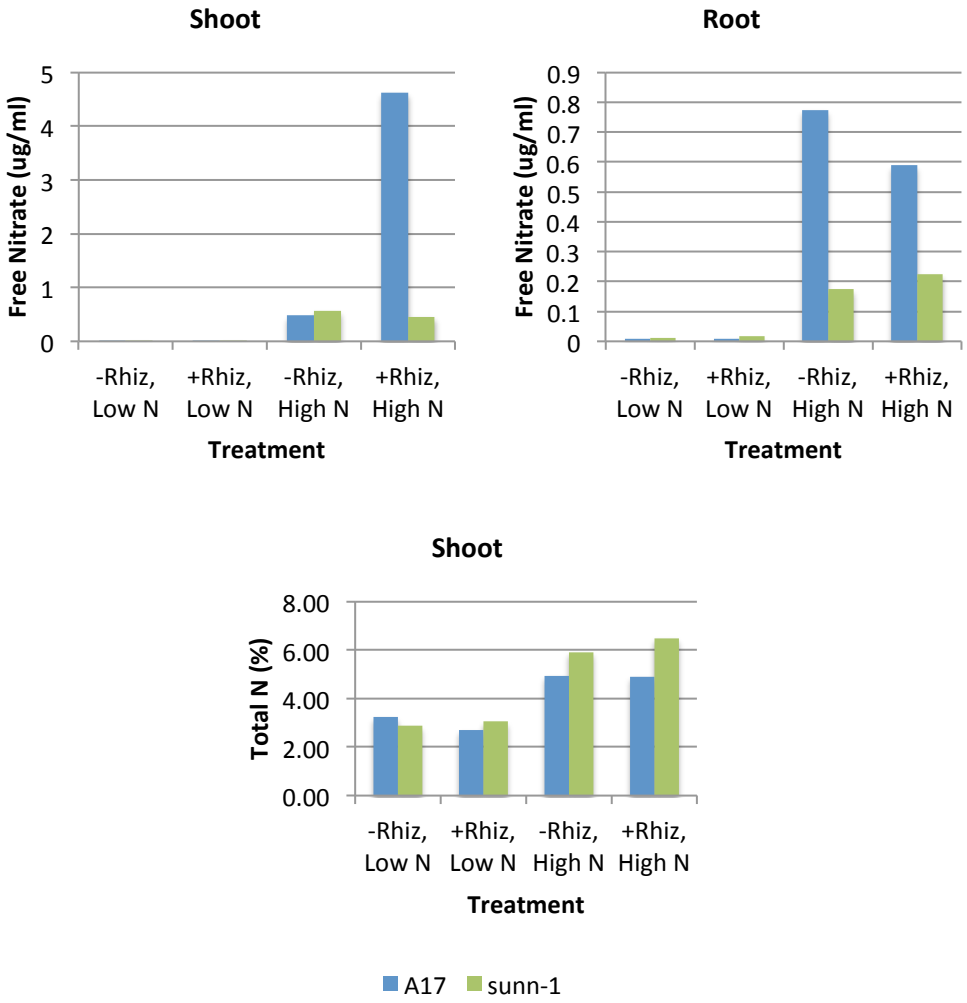


Figure 5-3: Free nitrate and total N in root and shoot of A17 (blue bars) and *sunn-1* (green bars) rhizobia inoculated (+Rhiz) or mock inoculated (-Rhiz) samples at low (0.1 mM) and high (5 mM) concentrations of N. Bars represent measured values for one biological replicate (n=54) thus no statistical test was performed on the data.

is because in hypernodulating mutants the nitrogen capacity per nodule is reduced. Hence, despite having a higher number of nodules compared to the wild type, they do not fix more nitrogen (Mortier et al., 2012b). In A17, free nitrate concentration was higher in the shoot in +Rhiz samples at high N compared to low N and compared to *sunn-1* (Figure 5-3). This could be one of the reasons for smaller root size (shorter PR and LR length with a smaller number of LRs) in the presence of rhizobia at high N. LR elongation and development is inhibited by the systemic signaling pathways controlling LR development based on shoot N status (Forde, 2014).

5.2 Whole genome expression profiling identifies rhizobia and *SUNN* regulated low and high N responses in A17 and *sunn-1*

The response of A17 and *sunn-1* to low and high N concentration and rhizobia is different. This response is more enhanced in +Rhiz *sunn-1* plants at low N (Figure 5-4). In low N *sunn-1* produces a significantly higher number of nodules but LR length is shorter compared to A17. Changes in A17 and *sunn-1* root architecture under low (0.1 mM) and high (5 mM) N suggest a regulatory effect for rhizobia on root architecture. It also suggests that *SUNN* could be involved in regulating LR length as also reported in other studies (Jin et al., 2012).

Genome-wide expression profiling (with microarrays) was used to study gene expression changes affect by low or high N and rhizobia inoculation in A17 and *sunn-1* grown under N deprivation (low N, 0.1 mM) condition (Figure 5-4). Rhizobia inoculated (+Rhiz) and mock-inoculated (-Rhiz) seedlings of A17 and *sunn-1* were grown at low N (NH_4NO_3 0.1 mM) then at 14 dpi treated with high (5 mM) or low (0.1 mM) N for 6 h (Figure 5-5 a). Analysis of the microarray data (Figure 5-5 b) identified a list of candidate genes regulating nodule number and LR development according to the plant N status, by implicating gene expression variation in the control of root and nodule phenotype variation between *sunn-1* and A17 (Figure 5-4).

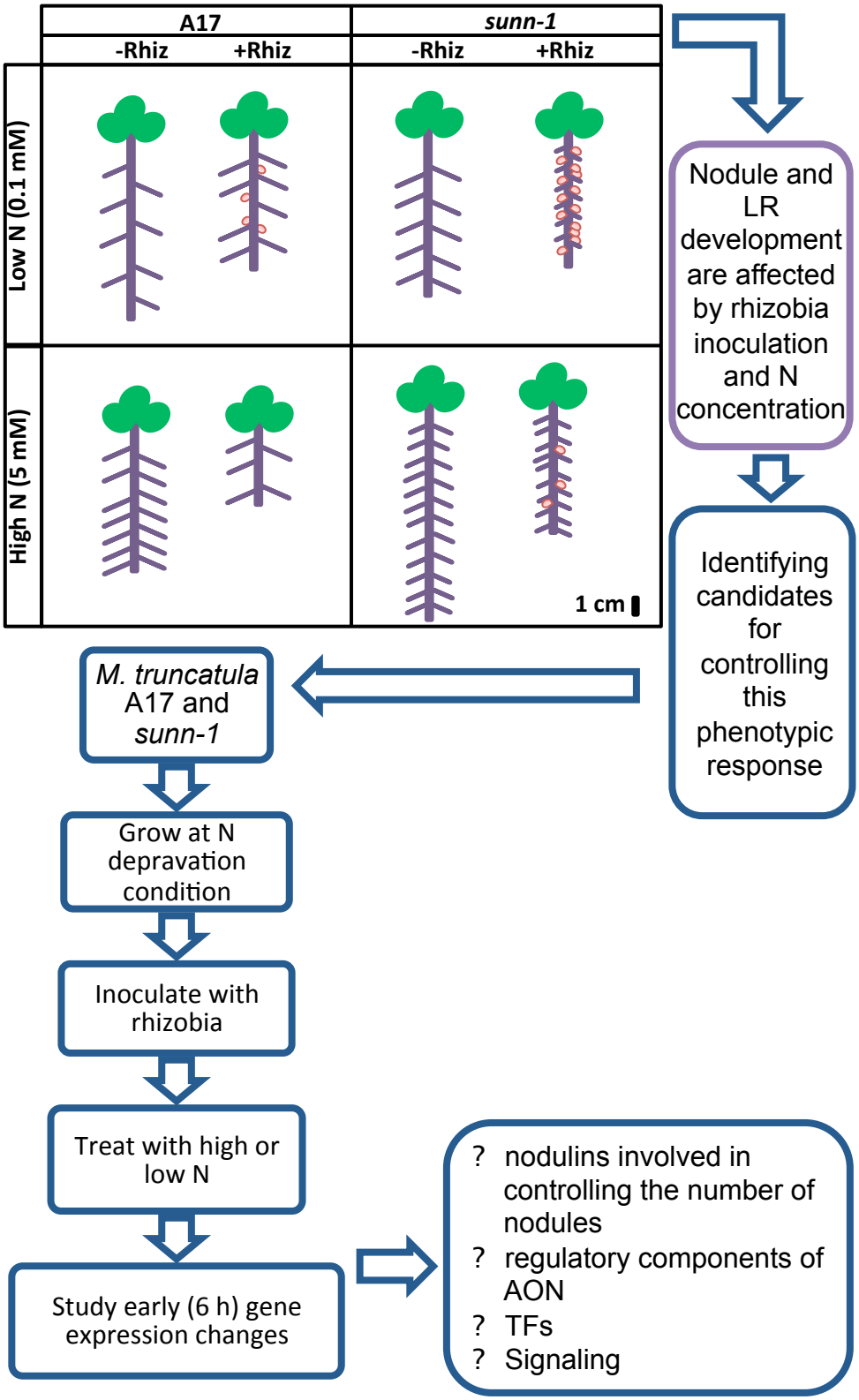
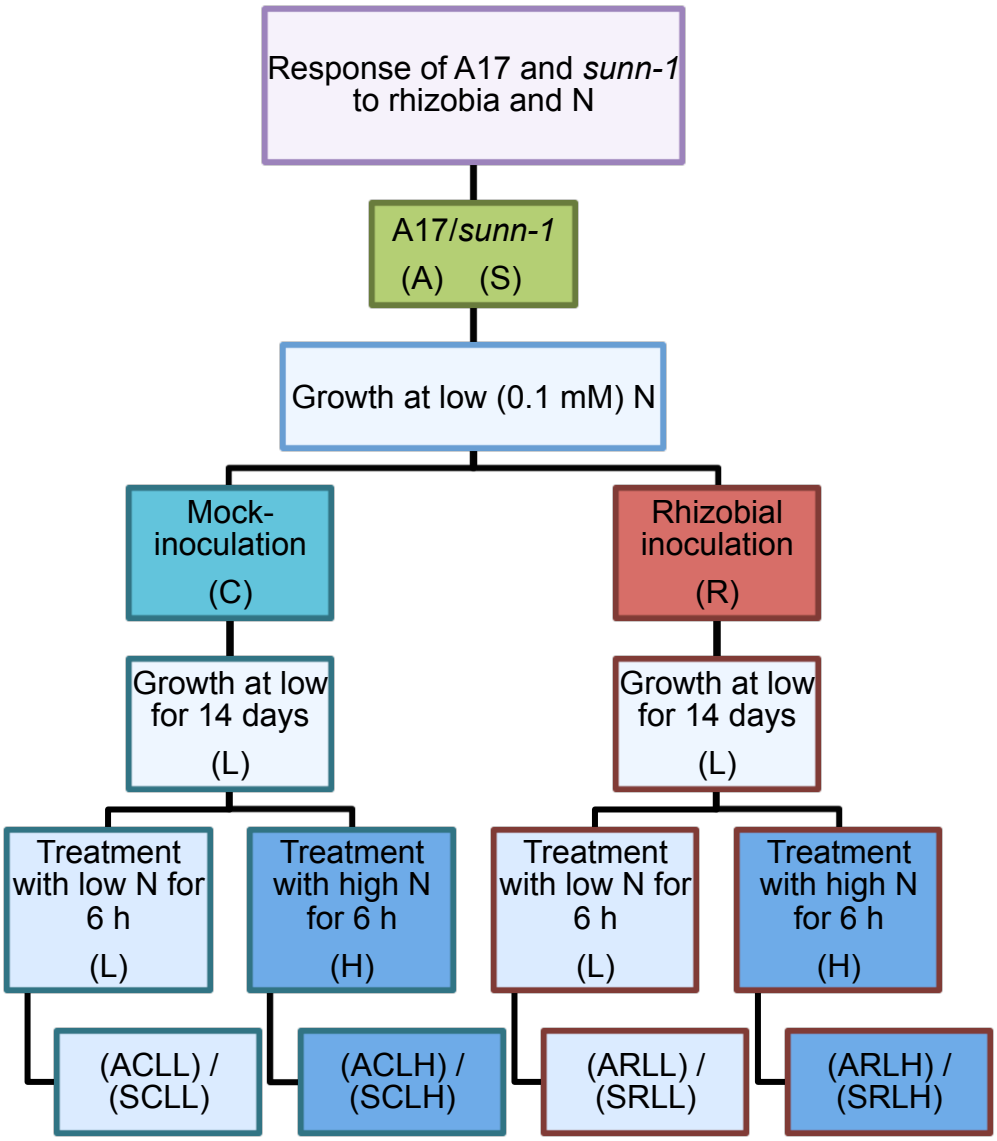


Figure 5-4: Schematic representation of interaction of rhizobia and N on root architecture in A17 and *sunn-1*. *sunn-1* mutants have a higher number of LRs but the average of LR length is shorter compared to A17. This effect is more pronounced in the presence of rhizobia on low N (nodulating) and high N. Differences between A17 and hypernodulating *sunn-1* mutants on low (0.1 mM) vs. high (5 mM) N; n=12 plants.

Figure 5-5

(a)


Response of A17 and *sunn-1* to rhizobia and N
A17/*sunn-1*
(A) (S)
Growth at low (0.1 mM) N
Mock-inoculation
(C)
Rhizobial inoculation
(R)
Growth at low for 14 days
(L)
Growth at low for 14 days
(L)
Treatment with low N for 6 h
(L)
Treatment with high N for 6 h
(H)
Treatment with low N for 6 h
(L)
Treatment with high N for 6 h
(H)
(ACLL) / (SCLL)
(ACLH) / (SCLH)
(ARLL) / (SRLL)
(ARLH) / (SRLH)

(b)

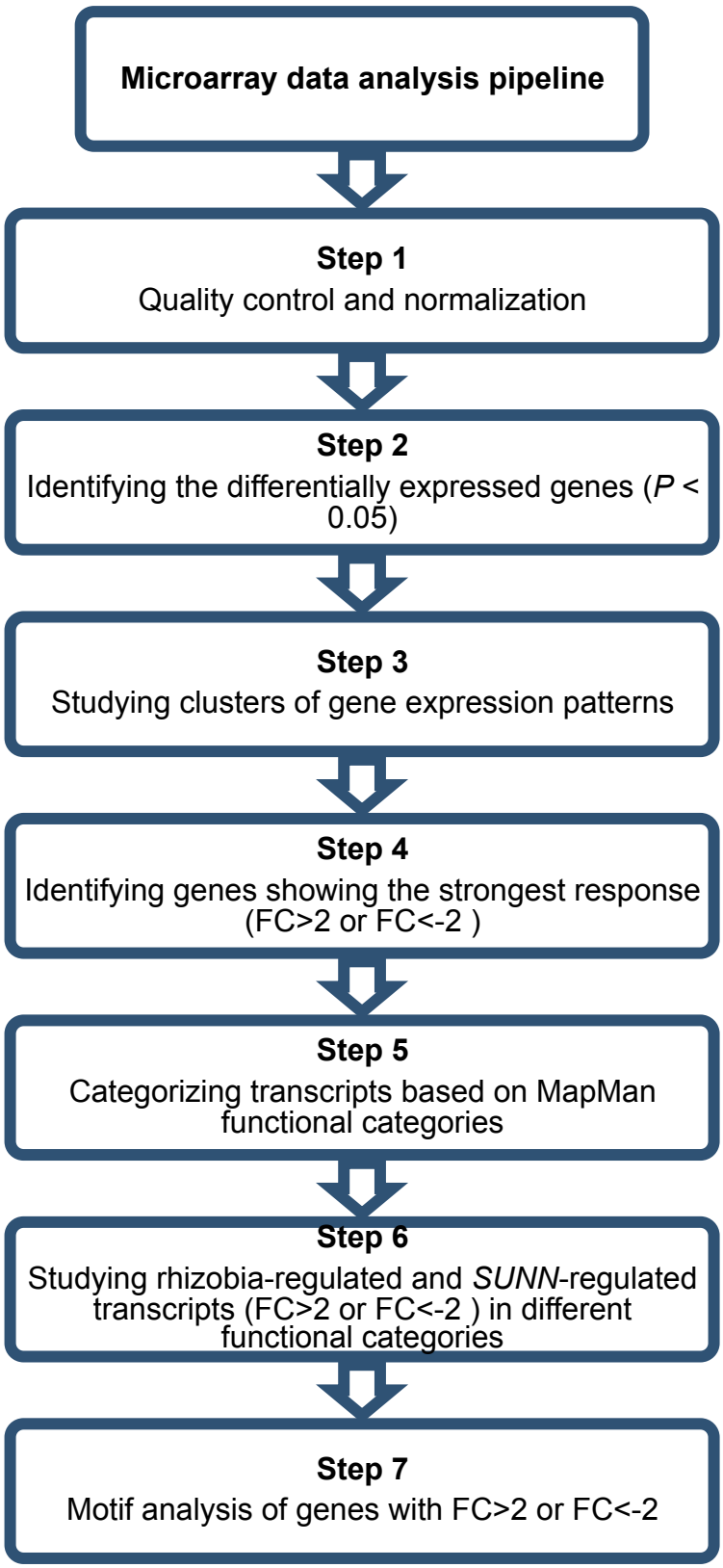


Figure 5-5: (a) Experimental design for studying whole genome expression changes 6 hours post treatment with high N (5 mM NH_4NO_3) or low N (0.1 mM NH_4NO_3) in rhizobia inoculated and mock inoculated A17 and *sunn-1* seedlings grown at low N. (b) Steps for analysing the microarray data and identifying significantly affected genes by the treatments.

5.2.1 Quality control and normalization

The steps taken for quality control and normalization of the data was as previously used (section 4.1). Samples in 3 biological replicates with 3 repeats for each biological replicate (n=6 plants per plate = 1 repeat) were treated as in Figure 5-5 a. Roots of 3 plants from each repeat were harvested for each biological replicate (n=9 plants). The labeled cDNA samples were assigned to the microarrays roughly at random. The distribution of the XYS expression values before and after normalization (using the RMA algorithm (Carvalho, 2010)) were plotted as box plots and smoothed histograms (Figure 5-6 a-h) to compare the distribution between the data sets for identifying the level of skewness and variability to gain an overview of the quality of the data. The histograms and boxplots for A17 and *sunn-1* showed that after normalization the data sets were following almost a similar distribution and there were no outliers.

Expression levels of the normalised data were compared (between replicates and across the data set) using Pearson's *r* correlation coefficients and the correlations were plotted as a heatmaps (Figure 5-6 i and j). The pair-wise comparisons of the data samples for A17 (Figure 5-6 i) and *sunn-1* (Figure 5-6 j) showed that there was a strong correlation between microarray replicate sets. This is indicated by predominant green coloration in the heatmaps (Figure 5-6 i and j) for a row or column with correlation ranging between $0.935 \leq r \leq 0.993$ with average $r = 0.972$ for A17 samples and $0.930 \leq r \leq 0.992$ with average $r = 0.965$ for *sunn-1* samples. This strong expression comparison of the replicates and arrays confirmed the good quality of the data hence all replicates were used for further analysis.

5.2.2 Identifying genes with significant expression changes

Differentially expressed genes were identified using the GaGa algorithm (Rossell, 2009). The groups each A17 or *sunn-1* samples belonged to (each in 3 replicates) were defined as in Figure 5-5 a. Patterns of comparisons between treatments were generated separately for the following groups (16

Figure 5-6

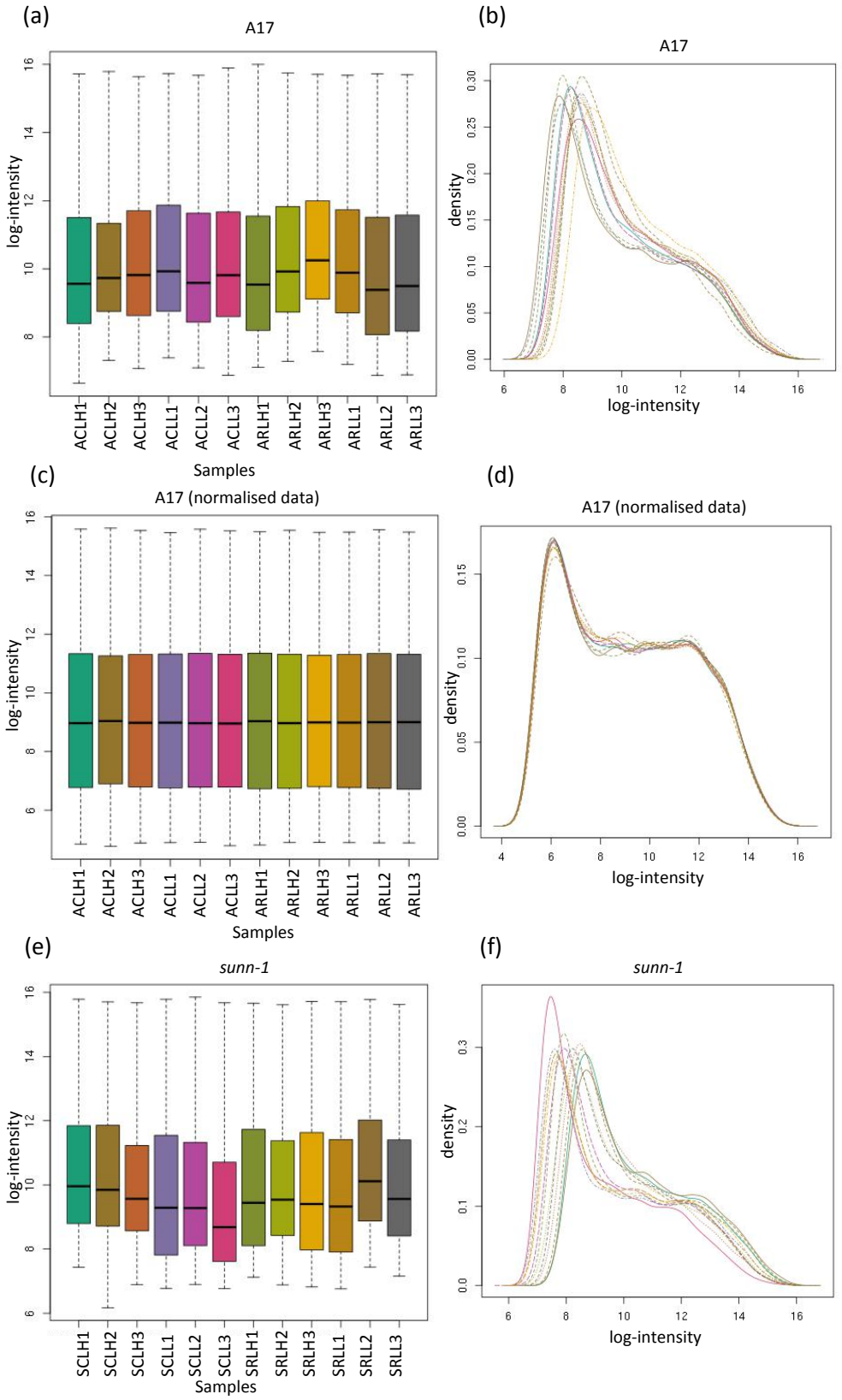
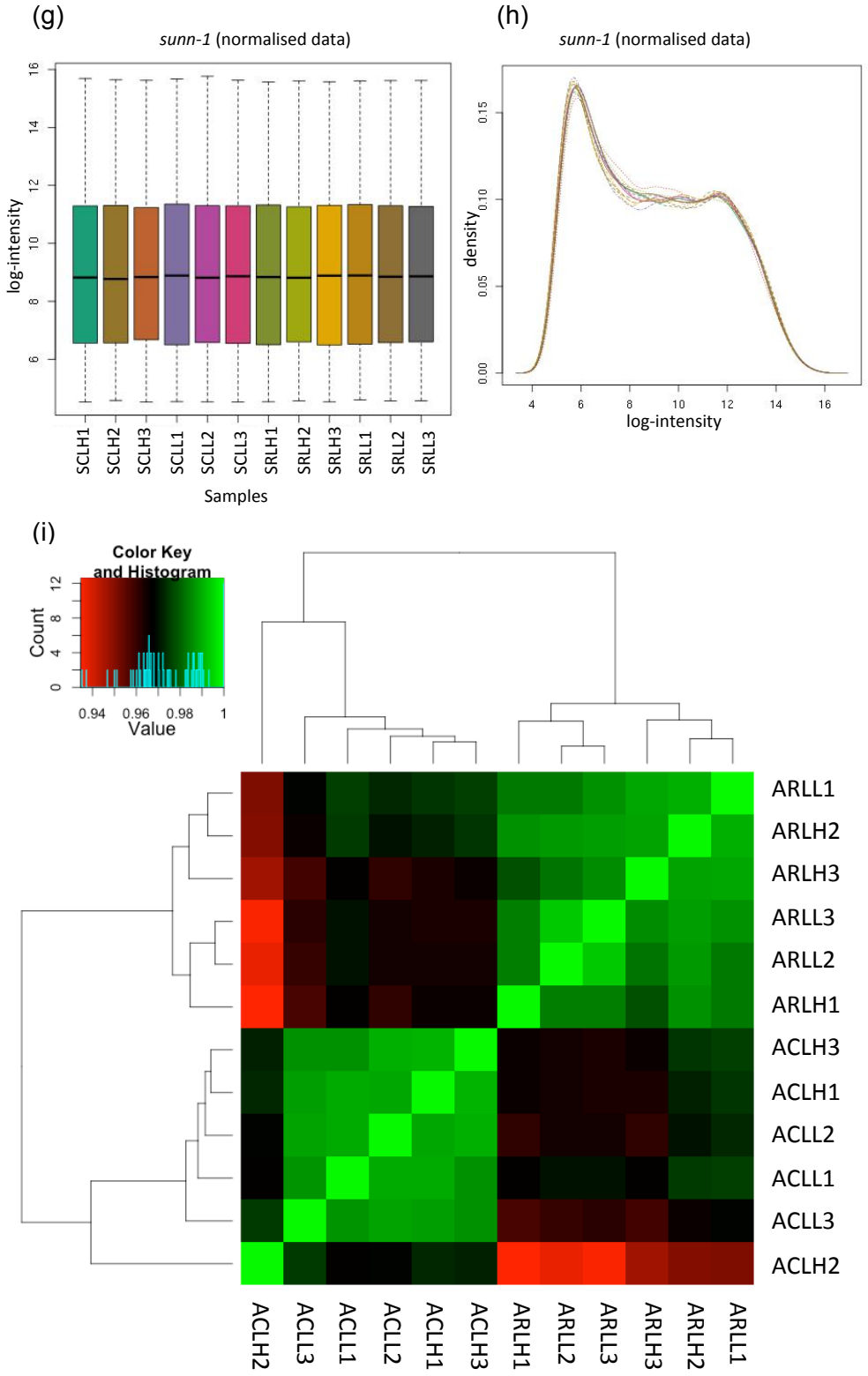

(a)
A17
log-intensity
Samples
ACLH1
ACLH2
ACLH3
ACLL1
ACLL2
ACLL3
ARLH1
ARLH2
ARLH3
ARLL1
ARLL2
ARLL3
(b)
A17
density
log-intensity
(c)
A17 (normalised data)
log-intensity
Samples
(d)
A17 (normalised data)
density
log-intensity
(e)
sunn-1
log-intensity
Samples
SCLH1
SCLH2
SCLH3
SCLL1
SCLL2
SCLL3
SRLH1
SRLH2
SRLH3
SRLL1
SRLL2
SRLL3
(f)
sunn-1
density
log-intensity

Figure 5-6, continue



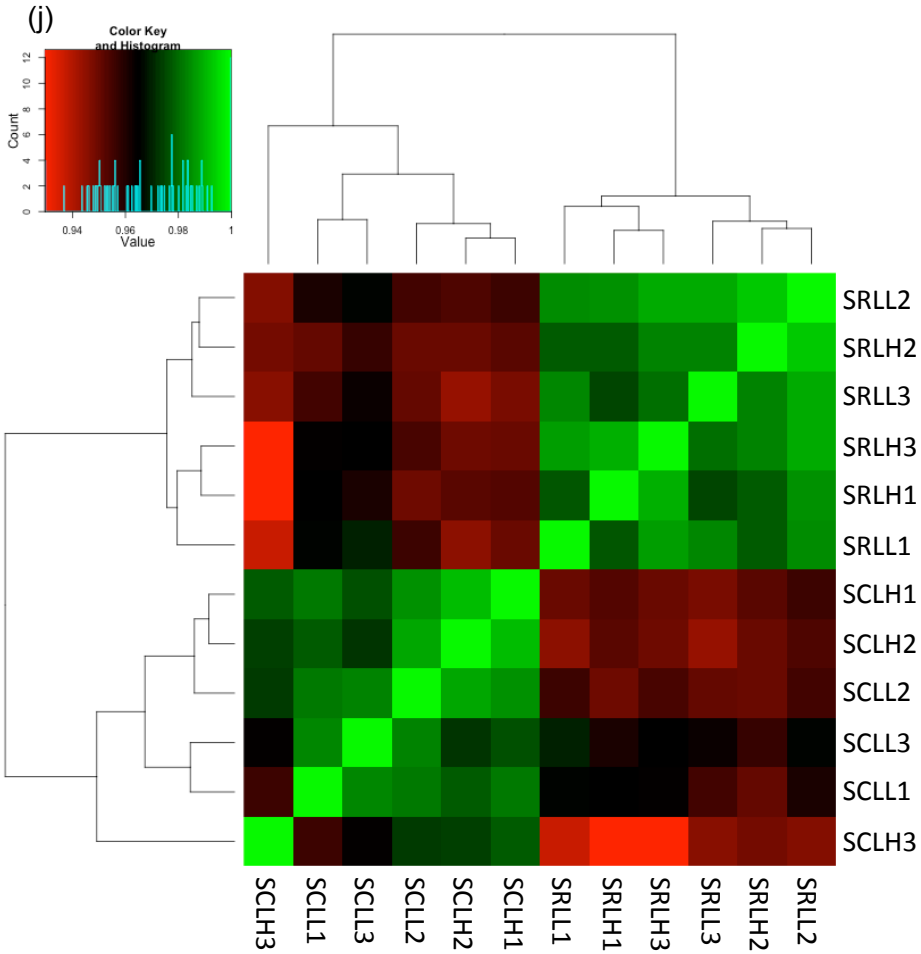


Figure 5-6: Distribution of log_2-intensities (XYS expression values) of 3 biological replicates of mock (C) or rhizobia (R) inoculated A17 (A) or *sunn-1* (S) samples treated with low (L) or high (H) N before and after normalisation plotted as box plots (a and c for A17, e and g for *sunn-1*) and smooth histograms (b and d for A17, f and h for *sunn-1*). i and j) Heatmaps showing the level of correlation between all replicates of A17 (i) and *sunn-1* sample. The heatmaps are symmetric about the diagonal, and reading by column or row is equivalent. The diagonal shows correlation between each sample and itself (= 1). Labels correlate to the experimental design in figure 5-3.

patterns for each group): low and high N treated -Rhiz and +Rhiz samples of A17 (i.e. ACLL, ACLH, ARLL, ARLH) and *sunn-1* (i.e. SCLL, SCLH, SRLL, SRLH); -Rhiz (i.e. ACLL, ACLH, SCLL, SCLL) or +Rhiz (ARLL, ARLH, SRLL, SRLH) samples of A17 and *sunn-1* treated with low or high N. A GaGa model with a significance cutoff of $P<0.05$ was then applied to each group separately to identify the genes differentially expressed under the experiment conditions. These lists were amalgamated to generate a list of differentially expressed genes responding to low or high N treatment in the absence or presence of rhizobia in A17 and *sunn-1* backgrounds.

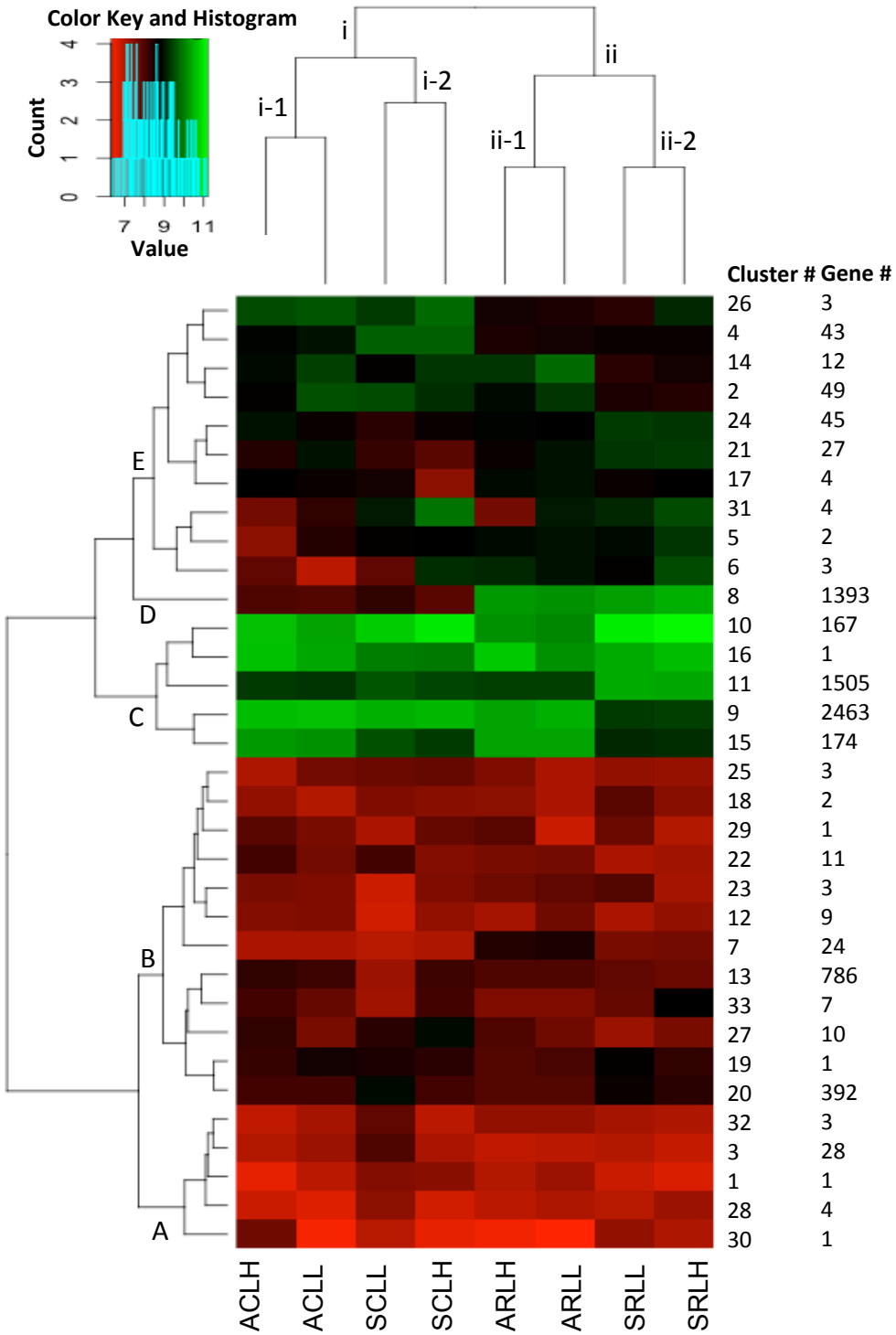
From a total of 64123 genes in the Mt3.5 gene model, 47529 genes were tested on the Nimblegen Mt3.5 custom expression array, of which 7186 genes showed significant gene expression changes under the experimental condition (Figure 5-5 a). This representing 15% of the genes on the array annotated in the *M. truncatula* 3.5 gene model.

5.2.3 Clusters of gene expression changes identifies a strong interaction between AON and rhizobia effects

An overview of the responses of A17 and *sunn-1* to rhizobia inoculation and low or high N treatment was gained by studying clusters of gene expression patterns. The RMA normalised expression values of the differential expressed genes were used to cluster these genes into 33 clusters (Figure 5-7). Silhouette statistics (Leonard and Peter, 1990) in MatLab was used to determine the number of clusters. This function takes various silhouette statistics for a series of sequentially increasing cluster partitions and plots them. The cluster partition with negative silhouette values minus 1 determines the number of clusters.

The average expression level within each cluster was then plotted as a heatmap (Figure 5-7 a). Patterns of gene expression changes in each cluster were studied to identify (1) the effect of high N treatment in the absence or presence of rhizobia in A17 and *sunn-1*, (2) whether the A17 and *sunn-1* responses to high N are similar or differ in -Rhiz and +Rhiz, (3) the effect

Figure 5-7, (a)


Color Key and Histogram
Count
Value
i
i-1
i-2
ii
ii-1
ii-2
Cluster # Gene #
26 3
4 43
14 12
2 49
24 45
21 27
17 4
31 4
5 2
6 3
8 1393
10 167
16 1
11 1505
9 2463
15 174
25 3
18 2
29 1
22 11
23 3
12 9
7 24
13 786
33 7
27 10
19 1
20 392
32 3
3 28
1 1
28 4
30 1
A
B
C
D
E
ACLH
ACLL
SCLL
SCLH
ARLH
ARLL
SRLL
SRLH

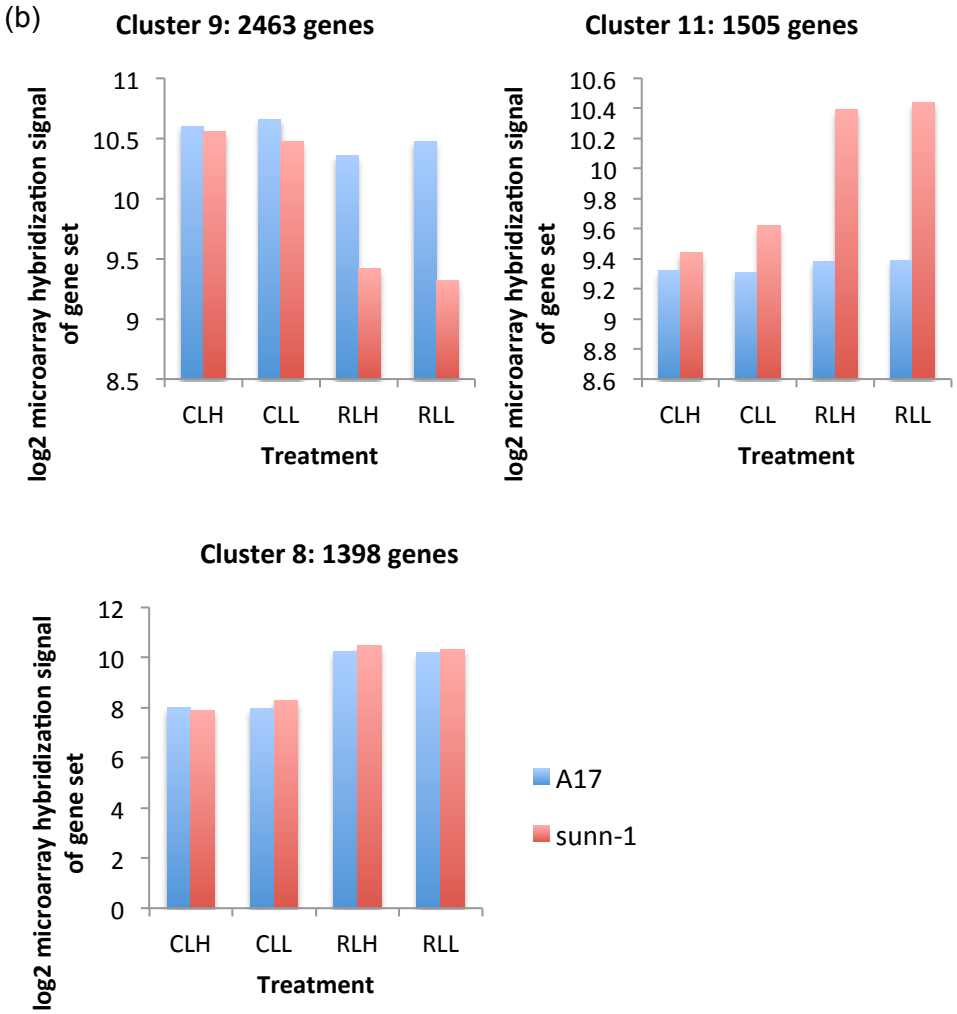


Figure 5-7: Expression patterns of clusters of genes differentially expressed in response to high (5 mM) or low (0.1 mM) N treatment and rhizobial inoculation in A17 and *sunn-1* whole root samples plotted as the average of $\log_2$ microarray hybridization signal of genes for each cluster. a) Heatmap showing expression patterns of all gene clusters (rows) at different experimental conditions (columns). Labels at the horizontal axis correlate to the experimental design in figure 5-5. b) Average of gene expression values in clusters 9, 8 and 11. Treatments at the horizontal axis: CLH (mock-inoculated and treated with high N), CLL (mock-inoculated and treated with low N), RLH (rhizobia inoculated and treated with high N), RLL (rhizobia inoculated and treated with low N).

of rhizobia inoculation at low N and high N, and how this might differ between A17 and *sunn-1* (4) the effect of *sunn-1* in -Rhiz or +Rhiz samples treated with high or low N.

The position of sample types in the clustering of all differentially expressed genes (Figure 5-7 a) shows that +Rhiz and -Rhiz samples of both A17 and *sunn-1* were clustered separately into two main clusters (branches i and ii in Figure 5-7 a) suggesting that rhizobia had a stronger effect on gene expression changes under the experiment conditions (Figure 5-5 a) than either genotype or N effect (branches i1-2 and ii1-2 in Figure 5-7 a). Within each +Rhiz and -Rhiz clade, low or high N treated A17 and *sunn-1* samples also formed separate clusters (branches i1-2 and ii1-2 in Figure 5-7 a) suggesting that the genotype effect was the next strongest. Hence, N showed to have a comparably more minor effect on gene expression changes of whole root samples of A17 and *sunn-1* under the experimental conditions.

The clustering on the vertical axis of the heatmap (Figure 5-7 a) also shows 5 main response patterns to rhizobia inoculation and low or high N treatment in A17 and *sunn-1* (branches A-E in figure 5-7 a). The average of gene expression levels is lower in the clusters in branches A and B compared to clusters in other branches. Branch C contains clusters with the highest expression levels compared to clusters in other branches. Interesting gene expression patterns in these clusters (for example clusters 9 and 11) could explain the phenotype observed in rhizobia and high N treated A17 and *sunn-1* samples.

Clusters in branch C mainly contain genes involved in N responses and LR and nodule developmental and regulatory pathways. As shown in the heatmap in Figure 5-7 a, in clusters 9 and 11 gene expression level is distinctively different in rhizobia inoculated *sunn-1* samples treated with low or high N from the other treated samples. This could indicate a *SUNN* effect in the presence of rhizobia and independent of N. As also observed in the phenotyping experiments that at low N *sunn-1* hypernodulated but developed

shorter LRs than A17 and at high N developed shorter LRs but more in number (Figure 5-1).

The gene expression behavior in cluster 9 affected by rhizobia, high or low N treatment in the +/-Rhiz and *SUNN* effect at these conditions could suit with finding candidate genes and could also explain the observed root phenotype (Figure 5-1). In this cluster with 2463 genes (Figure 5-7 b), A17 and *sunn-1* showed opposite responses to high N treatment in both -Rhiz and +Rhiz. The gene expression levels decreased in A17 in response to high N treatment both in -Rhiz and +Rhiz while it was increased in *sunn-1*. This is in agreement with the phenotypic experiments where RSA response to high N was different in seedlings of A17 and *sunn-1* (Figure 5-1). For cluster 9, +Rhiz samples of A17 and *sunn-1* gene expression level was lower than -Rhiz, both at low and high N. Suggesting that rhizobia had a similar effect on the trend of gene expression changes independent of genotype or N treatment. In this cluster the average of expression level is similar between A17 and *sunn-1* in non-nodulating (-Rhiz) samples, especially in high N, but *sunn-1* samples show a lower expression level than A17 in +Rhiz. This could suggest that the *SUNN* effect is stronger in rhizobia inoculated samples irrespective of the N condition and treatment.

In cluster 11 (1505 genes) (Figure 5-7 b), high N treatment seemed to have no significant effect on gene expression levels of +Rhiz or -Rhiz samples in either A17 or *sunn-1*. The rhizobia effect was similar in both A17 and *sunn-1* although *sunn-1* showed a stronger response to rhizobia. Genes in the cluster in *sunn-1* had a higher gene expression level compared to A17 in both low and high treatments and this was more prominent in the presence of rhizobia as shown in Figure 5-7 b. This may suggest a *SUNN* effect that is more dependent on the rhizobia effect than N effect.

In branch D cluster 8 (with 1398 genes) both A17 and *sunn-1* show distinctive clustering between +Rhiz and -Rhiz with higher gene expression levels in +Rhiz, indicative of a prominent rhizobia effect in this cluster that is

independent of N or genotype (Figure 5-7). The similar response to rhizobia appears to be independent of genotype or N effect. Gene expression levels are also the same between the two genotypes in +/-Rhiz at low or high N. The fact that the majority of the genes in this cluster are involved in the nodulation pathway (e.g. ENOD18, nodule inception protein and the nodule specific cysteine rich peptides) could indicate that this effect could be driven by nodulation genes.

5.2.4 Identifying genes having the strongest responses to rhizobia and low or high N treatment in A17 and *sunn-1*

To identify genes having the strongest responses to low or high N treatment or/and affected by rhizobia or/and genotype, FC of the expression values of the differentially expressed genes were calculated and genes with log2 FC of >2 or <-2 were identified. This was carried out to identify the (1) effect of high N treatment in the absence or presence of rhizobia, (2) rhizobia effect at low N (3) rhizobia effect at high N and (4) *SUNN* effect at low or high N in -Rhiz or +Rhiz samples (Table 5-3). To identify genes strongly affected by high N treatment (post N deprivation) the FC between ACLL and ACLH (high N effect in the absence of rhizobia in A17), ARLL and ARLH (high N effect in the presence of rhizobia in A17), SCLL and SCLH (high N effect in the absence of rhizobia in *sunn-1*), and SRLL and SRLH (high N effect in the presence of rhizobia in *sunn-1*) were calculated. For the effect of rhizobia at low N (i.e. N deprived samples treated with the same low N concentration) FC between ACLL and ARLL in A17, and SCLL and SRLL in *sunn-1* were calculated. The effect of rhizobia at high N (N deprived samples treated with high N) was studied by calculating FC between ACLH and ARLH in A17, and SCLH and SRLH in *sunn-1*. For the effect of *SUNN* in N deprived samples treated with the same low N concentration FC between ACLL and SCLL (for the absence of rhizobia) and ARLL and SRLL (for the presence of rhizobia) were calculated. FC between the absence of rhizobia (ACLH and SCLH) and the presence of rhizobia (ARLH and SRLH) were calculated to study the effect of *SUNN* in N deprived samples treated with high N (Table 5-3). The number

Table 5-3: Group of samples that were compared together to calculate FC. FC of the expression values of the differentially expressed genes between different samples (as indicated in the table) was calculated to identify genes responding to the effects listed in the table. Labels correlate to the experimental design in figure 5-5.

	FC calculated between samples	
Response	**A17**	***sunn-1***
High N effect in -Rhiz	Group 1: ACLL vs ACLH	Group 2: SCLL vs SCLH
High N effect in +Rhiz	Group 3: ARLL vs ARLH	Group 4: SRLL vs SRLH
Effect of rhizobia at low N	Group 5: ACLL vs ARLL	Group 6: SCLL vs SRLL
Effect of rhizobia at high N	Group 7: ACLH vs ARLH	Group 8: SCLH vs SRLH
SUNN effect at low N and -Rhiz	Group 9: ACLL vs SCLL	
SUNN effect at low N and +Rhiz	Group 10: ARLL vs SRLL	
SUNN effect at high N and -Rhiz	Group 11: ACLH vs SCLH	
SUNN effect at high N and +Rhiz	Group 12: ARLH vs SRLH	

of genes showing significant expression changes in response to the above effects is shown in Table 5-4.

Analysing of strongest FC responses showed that A17 and *sunn-1* had different response to high N in the absence or presence of rhizobia. However, as also observed in the cluster studies, the N effect was not strong compared to rhizobia and genotype effect. From 7186 genes that were identified as differentially expressed under the experimental conditions (Figure 5-5 a) 3 genes showed significant (FC>2 or FC<-2) expression changes in response to high N treatment in -Rhiz in A17 (with 100% being upregulated). This number was the same in +Rhiz samples but with a different N-response than -Rhiz (with 100% downregulated). The high N-response was different in *sunn-1* compared to A17 with a dominant high N-suppression (92% downregulated) and a higher number of differentially expressed genes (64 genes) showing significant expression changes (FC>2 or FC<-2) under high N treatment in -Rhiz. In +Rhiz no differentially expressed genes showed significant expression changes (FC>2 or FC<-2) in *sunn-1.*

Rhizobia regulated responses to low or high N was stronger in both A17 and *sunn-1* and a higher number of differentially expressed genes had strong (FC>2 or FC<-2) expression changes. Looking at the rhizobia regulated response at low N shows that, 574 differentially expressed genes were significantly (FC>2 or FC<-2) affected by rhizobia in A17 (99% induced) and 849 genes in *sunn-1* (80% induced). This number of regulated differentially expressed genes was almost the same (8% in A17 and 14% in *sunn-1*) at high N of which 100% were N-induced in A17 and 83% in *sunn-1*.

The number of N-regulated or rhizobia regulated genes in response to N in A17 and *sunn-1* (Table 5-5) indicates that the predominant effect was a rhizobia effect (independent of N) and that the (more minor) rhizobia-regulated response to N was stronger in *sunn-1* than A17. These all could explain the different root phenotype and nodulation behavior A17 and *sunn-1* show at low and high N in the absence and presence of rhizobia (Figure 5-1).

Table 5-4: The number of differentially expressed genes of A17 and *sunn-1* that show significant (FC>1 or FC<-1) expression changes in response to the effects in table 5-3.

Response	Number of significantly expressed genes (FC>2 or FC<-2)					
	A17			*sunn-1*		
	T	U	D	T	U	D
High N effect in -Rhiz	3	3	0	64	5	59
High N effect in +Rhiz	3	0	3	0	0	0
Effect of rhizobia at low N	574	567	7	849	679	170
Effect of rhizobia at high N	575	575	0	1004	828	176
	T		U		D	
SUNN effect at low N and -Rhiz	58		45		13	
SUNN effect at low N and +Rhiz	418		129		289	
SUNN effect at high N and -Rhiz	31		9		22	
SUNN effect at high N and +Rhiz	274		157		117	

T= Total, U= Upregulated, D= Downregulated

Studying the effect of *SUNN* at low or high N in the absence or presence of rhizobia again shows that the prominent rhizobia effect is independent of the N concentration. The number of significantly (FC>2 or FC<-2) affected differentially expressed genes was higher in +Rhiz samples both at low N (418, 31% upregulated) and high N (274 genes, 57% upregulated). This compares to the effect of *SUNN* in -Rhiz: 58 differentially expressed genes were significantly differentially expressed at low N (78% upregulated) with 31 genes significantly differentially expressed at high N (29% upregulated). The higher number of putative *SUNN*-regulated genes that was observed at low N in +Rhiz (418 genes), most of which were putatively repressed by *SUNN* (69%) could be linked/controlling the hypernodulating phenotype and short LRs observed at low N in +Rhiz samples (Figure 5-4). These genes may function in the pathways that regulate the number of nodules and balance LR development with nodulation. To evaluate this hypothesis, the following sections describe analysis of some of the putative regulators and TFs that may be involved in these regulatory pathways.

5.2.5 Rhizobia and *SUNN* mediated responses to low and high N affecting RSA

To interpret the genomic expression data in the context of known biological processes or pathways, groups of differentially expressed genes showing the strongest (FC>2 or FC<-2) rhizobia or/and *SUNN* mediated responses to low and high N in A17 and *sunn-1* (Tables 5-3 and 5-4) were assigned to different pathways and functional categories using the MapMan software (Thimm et al., 2004). This analysis used Wilcoxon test with Benjamini-Hochberg correction to identify functional categories (bins and sub-bins) statistically different from other categories. Based on this analysis, significantly different (P<0.05) functional categories (Table 5-5) that could be involved in rhizobia and *SUNN* responses to N were selected for further study. Additionally, some other functional categories (as in Figure 5-8) with a higher P value were also selected because of their abundance of genes and

Table 5-5: Rhizobia-regulated (a) and *SUNN*-regulated (b) biological categories as analysed by Mapman (Wilcoxon rank sum test with Benjamini-Hochberg corrected; $P < 0.05$). Elements= number of genes in each category, -Rhiz= absence of rhizobia (mock-inoculated), +Rhiz= presence of rhizobia (rhizobia inoculated); high N= treated with 5 mM N, low N= treated with low N (0.1 mM).

(a)

Rhizobia-regulated N responses				
	Bin	Name	Elements	p-value
A17 (High N)	1.1.1	PS.lightreaction.photosystem II	9	0.0432
	10	cell wall	127	0.0020
	10.5	cell wall.cell wall proteins	20	0.0158
	10.5.1	cell wall.cell wall proteins.AGPs	17	0.0577
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	0.0577
	16.1	secondary metabolism.simple phenols	13	0.0432
	20	stress	317	0.0054
	20.1	stress.biotic	251	0.0218
	21.3	redox.heme	13	5.1639E-06
	26	misc	388	0.0004
	26.1	misc.cytochrome P450	64	0.0577
	27	RNA	417	0.0020
	27.3	RNA.regulation of transcription	362	0.0183
	29	protein	442	0.0356
	30	signalling	418	0.0093
	30.2	signalling.receptor kinases	295	0.0226
sunn-1 (High N)	10	cell wall	127	1.3413E-11
	10.3	cell wall.hemicellulose synthesis	5	0.0582
	10.5	cell wall.cell wall proteins	20	0.0005
	10.5.1	cell wall.cell wall proteins.AGPs	17	0.0011
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	0.0011
	10.6	cell wall.degradation	47	0.0040
	10.8	cell wall.pectin*esterases	19	0.0186
	16	secondary metabolism	143	1.0371E-11
	16.1	secondary metabolism.simple phenols	13	0.0001
	16.1.5	secondary metabolism.isoprenoids.terpenoids	9	0.0218
	16.2	secondary metabolism.phenylpropanoids	53	1.5477E-07
	16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	20	0.0002
	16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	7	0.0246
	16.8	secondary metabolism.flavonoids	40	0.0001
	16.8.3	secondary metabolism.flavonoids.dihydroflavonols	17	0.0564
	17	hormone metabolism	159	0.0001

Table 5-5 a, continue

	Rhizobia-regulated N responses			
	Bin	Name	Elements	p-value
sunn-1 (High N)	17.2	hormone metabolism.auxin	42	0.0456
	17.4.2	hormone metabolism.cytokinin.signal transduction	5	0.0573
	20	stress	317	1.6603E-16
	20.1	stress.biotic	251	7.9077E-10
	20.1.7	stress.biotic.PR-proteins	144	0.0007
	20.2	stress.abiotic	67	7.9351E-07
	20.2.1	stress.abiotic.heat	26	0.0083
	20.2.99	stress.abiotic.unspecified	24	7.6132E-06
	21.2	redox.ascorbate and glutathione	11	0.0582
	21.3	redox.heme	13	8.1260E-06
	27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	26	0.0005
	27.3.40	RNA.regulation of transcription.Aux/IAA family	6	0.0224
	26	misc	388	1.9307E-30
	26.1	misc.cytochrome P450	64	4.5969E-10
	26.12	misc.peroxidases	43	1.5440E-05
	26.19	misc.plastocyanin-like	21	0.0251
	26.2	misc.UDP glucosyl and glucoronyl transferases	37	0.0001
	26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11	0.0075
	26.4	misc.beta 1,3 glucan hydrolases	24	0.0195
	26.9	misc.glutathione S transferases	11	0.0030
	28	DNA	117	0.0300
	30	signalling	418	5.2397E-20
	30.1	signalling.in sugar and nutrient physiology	9	0.0288
	30.1.1	signalling.in sugar and nutrient physiology	8	0.0113
	30.2	signalling.receptor kinases	295	5.3751E-20
	30.2.11	signalling.receptor kinases.leucine rich repeat XI	60	3.0406E-06

Table 5-5 a, continue

Rhizobia-regulated N responses				
	Bin	Name	Elements	p-value
sunn-1 (High N)	30.2.16	signalling.receptor kinases.Catharanthus roseus-like RLK1	6	0.0246
	30.2.17	signalling.receptor kinases.DUF 26	94	2.9030E-06
	30.2.20	signalling.receptor kinases.wheat LRK10 like	16	0.0067
	30.2.24	signalling.receptor kinases.S-locus glycoprotein like	31	3.7143E-06
	34.14	transport.unspecified cations	13	0.0456
A17 (Low N)	16	secondary metabolism	143	0.0067
	16.1.5	secondary metabolism.isoprenoids.terpenoids	9	0.0089
	16.2	secondary metabolism.phenylpropanoids	53	0.0401
	16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	7	0.0018
	20	stress	317	3.7023E-05
	20.1	stress.biotic	251	3.7023E-05
	20.1.7	stress.biotic.PR-proteins	144	0.0089
	21.3	redox.heme	13	1.3615E-06
	27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	26	0.0008
	26	misc	388	1.1974E-05
	26.1	misc.cytochrome P450	64	3.9342E-09
	26.2	misc.UDP glucosyl and glucoronyl transferases	37	0.0143
	26.9	misc.glutathione S transferases	11	0.0448
	30	signalling	418	3.4703E-09
	30.2	signalling.receptor kinases	295	2.0574E-13
	30.2.11	signalling.receptor kinases.leucine rich repeat XI	60	0.0370
	30.2.17	signalling.receptor kinases.DUF 26	94	3.7023E-05
	30.2.24	signalling.receptor kinases.S-locus glycoprotein like	31	0.0008
	34.13	transport.peptides and oligopeptides	41	0.0307
sunn-1 (Low N)	10	cell wall	127	9.1331E-13
	10.5	cell wall.cell wall proteins	20	1.2785E-05
	10.5.1	cell wall.cell wall proteins.AGPs	17	2.8386E-05
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	2.8386E-05

Table 5-5 a, continue

Rhizobia-regulated N responses				
	Bin	Name	Elements	p-value
sunn-1 (Low N)	10.6	cell wall.degradation	47	0.0214
	10.8	cell wall.pectin*esterases	19	0.0048
	10.8.99	cell wall.pectin*esterases.misc	5	0.0359
	11.9	lipid metabolism.lipid degradation	31	0.0320
	16	secondary metabolism	143	7.1836E-12
	16.1	secondary metabolism.simple phenols	13	0.0003
	16.1.5	secondary metabolism.isoprenoids.terpenoids	9	0.0017
	16.2	secondary metabolism.phenylpropanoids	53	5.9151E-08
	16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	20	0.0002
	16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	7	0.0111
	16.8	secondary metabolism.flavonoids	40	0.0002
	16.8.3	secondary metabolism.flavonoids.dihydroflavonols	17	0.0110
	17	hormone metabolism	159	8.4534E-08
	17.2	hormone metabolism.auxin	42	0.0017
	17.2.3	hormone metabolism.auxin.induced-regulated-responsive-activated	36	0.0085
	17.5	hormone metabolism.ethylene	42	0.0014
	17.5.2	hormone metabolism.ethylene.signal transduction	12	0.0544
	20	stress	317	4.4091E-25
	20.1	stress.biotic	251	3.2302E-17
	20.1.7	stress.biotic.PR-proteins	144	8.7498E-08
	20.2	stress.abiotic	67	2.4818E-07
	20.2.1	stress.abiotic.heat	26	0.0075
	20.2.99	stress.abiotic.unspecified	24	5.7294E-06
	21.2	redox.ascorbate and glutathione	11	0.0558
	21.3	redox.heme	13	1.3589E-05
	26	misc	388	4.2875E-34
	26.1	misc.cytochrome P450	64	3.3169E-11
	26.1	misc.misc2	16	0.0570
	26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	12	0.0524

Table 5-5 a, continue

	Rhizobia-regulated N responses			
	Bin	Name	Elements	p-value
sunn-1 (Low N)	26.12	misc.peroxidases	43	4.5711E-09
	26.19	misc.plastocyanin-like	21	0.0514
	26.2	misc.UDP glucosyl and glucoronyl transferases	37	0.0001
	26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11	0.0312
	26.4	misc.beta 1,3 glucan hydrolases	24	0.0085
	26.9	misc.glutathione S transferases	11	0.0005
	27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	26	0.0002
	27.3.40	RNA.regulation of transcription.Aux/IAA family	6	0.0171
	30	signalling	418	3.4985E-25
	30.1	signalling.in sugar and nutrient physiology	9	0.0110
	30.1.1	signalling.in sugar and nutrient physiology	8	0.0036
	30.2	signalling.receptor kinases	295	8.9984E-25
	30.2.11	signalling.receptor kinases.leucine rich repeat XI	60	1.6711E-07
	30.2.16	signalling.receptor kinases.Catharanthus roseus-like RLK1	6	0.0514
	30.2.17	signalling.receptor kinases.DUF 26	94	7.2292E-07
	30.2.20	signalling.receptor kinases.wheat LRK10 like	16	0.0017
	30.2.24	signalling.receptor kinases.S-locus glycoprotein like	31	2.8813E-07
	31	cell	135	0.0065
	34.14	transport.unspecified cations	13	0.0085

(b)

	SUNN regulated N responses			
	Bin	Name	Elements	p-value
-Rhiz (High N)	10	cell wall	127	4.8502E-07
	10.5	cell wall.cell wall proteins	20	0.0002
	10.5.1	cell wall.cell wall proteins.AGPs	17	4.2896E-06
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	4.2896E-06
	10.7	cell wall.modification	15	0.0447
	10.8.99	cell wall.pectin*esterases.misc	5	0.0159
	17.4.2	hormone metabolism.cytokinin.signal transduction	5	0.0390
	21.3	redox.heme	13	0.0364

Table 5-5 b, continue

	***SUNN* regulated N responses**			
	Bin	**Name**	**Elements**	**p-value**
-Rhiz (High N)	26.1	misc.cytochrome P450	64	0.0025
	26.12	misc.peroxidases	43	0.0479
	26.2	misc.UDP glucosyl and glucoronyl transferases	37	0.0165
	26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11	0.0447
	27.3.21	RNA.regulation of transcription.GRAS transcription factor family	6	0.0447
	28.1.3	DNA.synthesis/chromatin structure.histone	13	0.0134
	30.2	signalling.receptor kinases	295	0.0046
	30.2.3	signalling.receptor kinases.leucine rich repeat III	10	0.0165
	30.2.17	signalling.receptor kinases.DUF 26	94	0.0020
	30.2.20	signalling.receptor kinases.wheat LRK10 like	16	0.0159
	31	cell	135	0.0068
	31.1	cell.organisation	87	0.0124
	31.3	cell.cycle	21	0.0165
+Rhiz (High N)	1.1	PS.lightreaction	24	0.0445
	10	cell wall	127	9.9397E-19
	10.2.1	cell wall.cellulose synthesis.cellulose synthase	12	0.0340
	10.5	cell wall.cell wall proteins	20	7.2740E-06
	10.5.1	cell wall.cell wall proteins.AGPs	17	1.5731E-06
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	1.5731E-06
	10.6	cell wall.degradation	47	8.2037E-06
	10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	31	0.0005
	10.7	cell wall.modification	15	0.0186
	10.8	cell wall.pectin*esterases	19	0.0130
	10.8.99	cell wall.pectin*esterases.misc	5	0.0373
	11.9.4.13	lipid metabolism.lipid degradation.beta-oxidation.acyl CoA reductase	4	0.0483
	13.1.4	amino acid metabolism.synthesis.branched chain group	6	0.0371
	13.1.4.5	amino acid metabolism.synthesis.branched chain group.isoleucine specific	5	0.0161
	16	secondary metabolism	143	5.1603E-08
	16.1	secondary metabolism.simple phenols	13	0.0005
	16.1.1	secondary metabolism.isoprenoids.non-mevalonate pathway	4	0.0161
	16.1.1.1	secondary metabolism.isoprenoids.non-mevalonate pathway.DXS	3	0.0483

Table 5-5 b, continue

	***SUNN* regulated N responses**			
	Bin	**Name**	**Elements**	**p-value**
+Rhiz (High N)	16.1.5	secondary metabolism.isoprenoids.terpenoids	9	0.0445
	16.2	secondary metabolism.phenylpropanoids	53	1.0966E-05
	16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	20	0.0038
	16.8	secondary metabolism.flavonoids	40	0.0002
	16.8.3	secondary metabolism.flavonoids.dihydroflavonols	17	0.0302
	17	hormone metabolism	159	0.0008
	17.6.3	hormone metabolism.gibberelin.induced-regulated-responsive-activated	8	0.0095
	20	stress	317	6.9087E-10
	20.1	stress.biotic	251	0.0001
	20.1.7	stress.biotic.PR-proteins	144	0.0264
	20.2	stress.abiotic	67	1.7774E-06
	20.2.1	stress.abiotic.heat	26	0.0356
	20.2.99	stress.abiotic.unspecified	24	1.7707E-06
	21	redox	41	0.0013
	26	misc	388	5.2507E-24
	26.1	misc.cytochrome P450	64	0.0002
	26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	12	0.0341
	26.12	misc.peroxidases	43	2.9638E-08
	26.19	misc.plastocyanin-like	21	0.0356
	26.2	misc.UDP glucosyl and glucoronyl transferases	37	0.0330
	26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11	0.0001
	26.4	misc.beta 1,3 glucan hydrolases	24	0.0218
	26.7	misc.oxidases - copper, flavone etc.	28	0.0214
	27	RNA	417	0.0119
	27.1.1	RNA.processing.splicing	11	0.0522
	27.2	RNA.transcription	11	0.0483
	27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	10	0.0490
	27.3.40	RNA.regulation of transcription.Aux/IAA family	6	0.0380
	28	DNA	117	0.0044
	28.99	DNA.unspecified	44	0.0002
	29	protein	442	0.0025
	29.5.11	protein.degradation.ubiquitin	132	0.0340
	29.5.11.4	protein.degradation.ubiquitin.E3	113	0.0121

Table 5-5 b, continue

	***SUNN* regulated N responses**			
	Bin	**Name**	**Elements**	**p-value**
+Rhiz (High N)	29.5.11.4.3	protein.degradation.ubiquitin.E3.SCF	64	0.0063
	29.5.11.4.3.2	protein.degradation.ubiquitin.E3.SCF.FBOX	55	0.0341
	30	signalling	418	3.5697E-12
	30.1.1	signalling.in sugar and nutrient physiology	8	0.0380
	30.2	signalling.receptor kinases	295	4.1621E-10
	30.2.11	signalling.receptor kinases.leucine rich repeat XI	60	0.0005
	30.2.17	signalling.receptor kinases.DUF 26	94	0.0302
	30.2.21	signalling.receptor kinases.lysine motif	9	0.0029
	30.2.24	signalling.receptor kinases.S-locus glycoprotein like	31	1.7774E-06
	30.3	signalling.calcium	40	0.0380
	31.3	cell.cycle	21	0.0345
-Rhiz (Low N)	10	cell wall	127	5.2416E-07
	10.5	cell wall.cell wall proteins	20	0.0473
	10.5.1	cell wall.cell wall proteins.AGPs	17	0.0177
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	0.0177
	10.6	cell wall.degradation	47	0.0177
	10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	31	0.0344
	10.7	cell wall.modification	15	0.0344
	16	secondary metabolism	143	0.0053
	16.1	secondary metabolism.simple phenols	13	0.0177
	20.1	stress.biotic	251	0.0495
	20.1.7	stress.biotic.PR-proteins	144	0.0177
	20.2	stress.abiotic	67	0.0294
	20.2.99	stress.abiotic.unspecified	24	0.0177
	21.1	redox.thioredoxin	10	0.0500
	21.3	redox.heme	13	0.0007
	26	misc	388	3.1469E-05
	26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11	0.0387
	28	DNA	117	0.0495
	28.99	DNA.unspecified	44	0.0033
	29.5.3	protein.degradation.cysteine protease	19	0.0187
	33.2	development.late embryogenesis abundant	10	0.0184
	34.13	transport.peptides and oligopeptides	41	0.0495
	35	not assigned	3819	0.0177
	35.2	not assigned.unknown	3819	0.0177

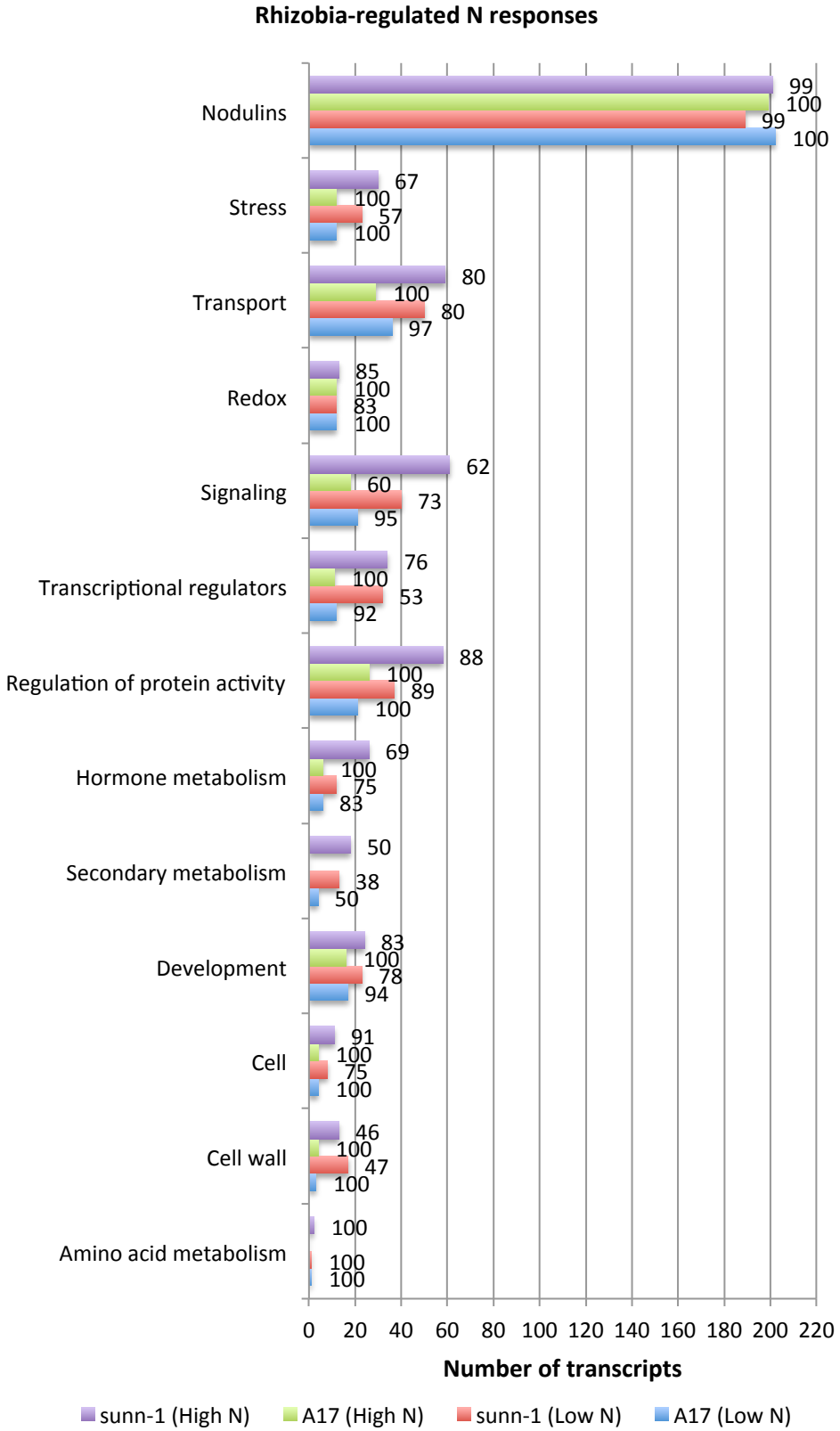
Table 5-5 b, continue

	***SUNN* regulated N responses**			
	Bin	**Name**	**Elements**	**p-value**
+Rhiz (Low N)				6.7202E
	10	cell wall	127	-17
	10.2	cell wall.cellulose synthesis	13	0.0546
	10.2.1	cell wall.cellulose synthesis.cellulose synthase	12	0.0304
				2.7838E
	10.5	cell wall.cell wall proteins	20	-06
				5.6185E
	10.5.1	cell wall.cell wall proteins.AGPs	17	-07
				5.6185E
	10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	17	-07
	10.6	cell wall.degradation	47	0.0003
	10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	31	0.0045
	10.7	cell wall.modification	15	0.0225
	10.8	cell wall.pectin*esterases	19	0.0547
	11.9	lipid metabolism.lipid degradation	31	0.0176
	11.9.3.2	lipid metabolism.lipid degradation.lysophospholipases.carboxylesterase	4	0.0370
	13.1.4.5	amino acid metabolism.synthesis.branched chain group.isoleucine specific	5	0.0306
				5.9850E
	16	secondary metabolism	143	-09
				1.1928E
	16.1	secondary metabolism.simple phenols	13	-05
	16.1.1	secondary metabolism.isoprenoids.non-mevalonate pathway	4	0.0167
	16.1.1.1	secondary metabolism.isoprenoids.non-mevalonate pathway.DXS	3	0.0491
				1.6665E
	16.2	secondary metabolism.phenylpropanoids	53	-06
	16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	20	0.0063
	16.8	secondary metabolism.flavonoids	40	0.0006
	16.8.3	secondary metabolism.flavonoids.dihydroflavonols	17	0.0176
				4.6217E
	17	hormone metabolism	159	-06
	17.2	hormone metabolism.auxin	42	0.0547
	17.5	hormone metabolism.ethylene	42	0.0293
	17.5.2	hormone metabolism.ethylene.signal transduction	12	0.0335
	17.6.3	hormone metabolism.gibberelin.induced-regulated-responsive-activated	8	0.0295
				1.0317E
	20	stress	317	-10

Table 5-5 b, continue

	***SUNN* regulated N responses**			
	Bin	**Name**	**Elements**	**p-value**
+Rhiz (Low N)	20.1	stress.biotic	251	0.0002
	20.2	stress.abiotic	67	5.9850E-09
	20.2.1	stress.abiotic.heat	26	0.0134
	20.2.99	stress.abiotic.unspecified	24	3.3848E-07
	21	redox	41	2.9683E-05
	21.1	redox.thioredoxin	10	0.0491
	21.3	redox.heme	13	0.0546
	26	misc	388	2.2420E-31
	26.1	misc.cytochrome P450	64	7.6940E-06
	26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	12	0.0356
	26.12	misc.peroxidases	43	5.9850E-09
	26.16	misc.myrosinases-lectin-jacalin	10	0.0167
	26.18	misc.invertase/pectin methylesterase inhibitor family protein	7	0.0528
	26.19	misc.plastocyanin-like	21	0.0066
	26.2	misc.UDP glucosyl and glucoronyl transferases	37	0.0299
	26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11	0.0006
	26.4	misc.beta 1,3 glucan hydrolases	24	0.0040
	26.7	misc.oxidases - copper, flavone etc.	28	0.0306
	26.9	misc.glutathione S transferases	11	0.0042
	27	RNA	417	0.0358
	27.1	RNA.processing	33	0.0358
	27.1.1	RNA.processing.splicing	11	0.0333
	27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	10	0.0479
	27.3.40	RNA.regulation of transcription.Aux/IAA family	6	0.0333
	28	DNA	117	0.0004
	28.99	DNA.unspecified	44	5.8143E-06
	30	signalling	418	5.9850E-09
	30.1.1	signalling.in sugar and nutrient physiology	8	0.0491
	30.2	signalling.receptor kinases	295	4.6217E-06
	30.2.11	signalling.receptor kinases.leucine rich repeat XI	60	0.0063
	30.2.21	signalling.receptor kinases.lysine motif	9	0.0023
	30.2.24	signalling.receptor kinases.S-locus glycoprotein like	31	0.0002
	30.3	signalling.calcium	40	0.0046
	31	cell	135	0.0306
	33.2	development.late embryogenesis abundant	10	0.0547
	34.7	transport.phosphate	12	0.0547

Figure 5-8, (a)


Rhizobia-regulated N responses
Nodulins
99
100
99
100
Stress
67
100
57
100
Transport
80
100
80
97
Redox
85
100
83
100
Signaling
62
60
73
95
Transcriptional regulators
76
100
53
92
Regulation of protein activity
88
100
89
100
Hormone metabolism
69
100
75
83
Secondary metabolism
50
38
50
Development
83
100
78
94
Cell
91
100
75
100
Cell wall
46
100
47
100
Amino acid metabolism
100
100
100
0 20 40 60 80 100 120 140 160 180 200 220
Number of transcripts
sunn-1 (High N)
A17 (High N)
sunn-1 (Low N)
A17 (Low N)

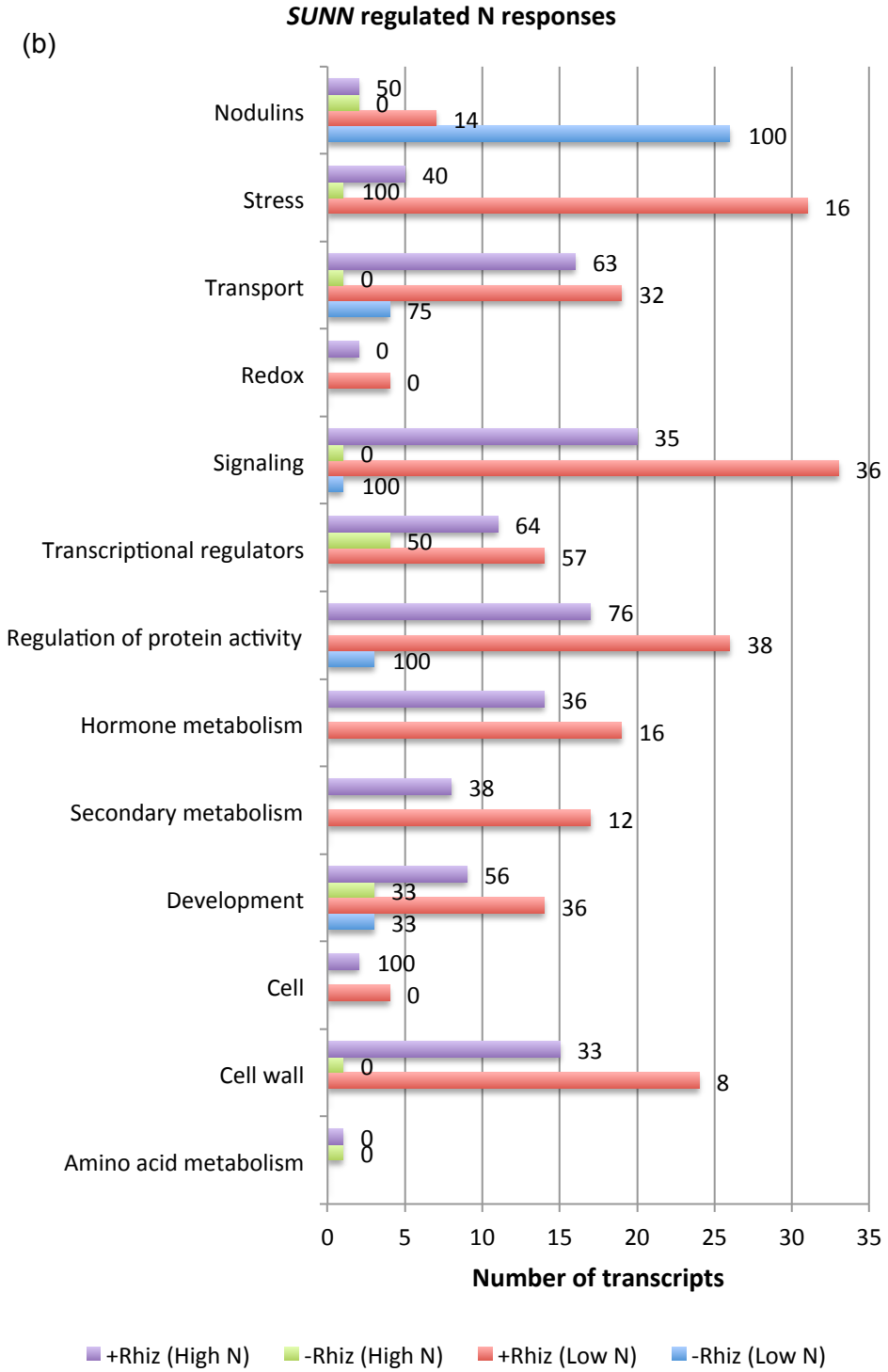


Figure 5-8: Main biological functional categories of the genes showing strong (a) rhizobia-regulated or (b) *SUNN*-regulated response to low and high N in A17 and *sunn-1* (FC >2 or FC <-2) defined by MapMan software (Thimm et al., 2004). The horizontal axis shows the number of genes in each category and numbers on top of each bar is the percentage of upregulated genes. The nodulin category is not one of the default functional categories in MapMan and all nodulins are assigned to this category manually. Table 5-5 contains Wilcoxon rank sum test *P* values for each functional category (Functional categories with $P > 0.05$ are not included in the table). -Rhiz= absence of rhizobia (mock-inoculated), +Rhiz= presence of rhizobia (rhizobia inoculated); high N= treated with 5 mM N, low N= treated with low N (0.1 mM).

significance in rhizobia and *SUNN* responses to N (as they will be discussed further in this section).

Based on the abundance of genes in MapMan functional categories, the main rhizobia regulated N responses belonged to nodulation, development, transport and regulatory functions including regulation of protein activity, regulation of RNA transcription and signaling (Figure 5-8 a). All nodulins that by default were not assigned to a known functional category by MapMan were included in a new category named nodulins (Figure 5-8). *SUNN* regulated responses to N were mainly stronger (higher number of differentially expressed genes with FC>2 or FC<-2) in the presence of rhizobia and especially at low N (Figure 5-8 b). Based on the abundance of genes in each category it appears that regulation of protein activity, regulation of RNA transcription, hormone metabolism and signaling was among the strongest responses regulated by *SUNN*.

5.2.5.1 Putative components of regulatory pathways balancing nodule number with LR development according to N availability

The phenotypic analysis presented earlier in this chapter suggests that the *sunn-1* mutant is a suitable candidate for studying the balance between nodule organogenesis and LR development in *Medicago*. At low N an interaction between regulation of nodule number and LR development was found in *sunn-1*: resource/carbon use for hypernodulation is putatively balanced by production of shorter LRs. At high N, *sunn-1* plants still have the ability to nodulate unlike in A17 where nodulation is inhibited, however the number of nodules is significantly less at high N. LR development also shows some differences between A17 and *sunn-1* at high N with shorter but more LRs in *sunn-1*.

Combined with the expression data analysis of significantly responding genes, we propose that under low N a mechanism exists to balance nodules and LRs, mediated by *sunn-1*. Here we try to identify some of the genes that may be involved in this balance by identifying differentially expressed genes

that show significant (FC>2 or FC<-2) rhizobia mediated expression changes and also by studying the differentially expressed genes that are strongly (FC>2 or FC<-2) affected by SUNN in low N. To provide a comparison between low and high N, responses at high N are also studied.

In both low and high N a higher number of differentially expressed genes showed significant (FC>2 or FC<-2) rhizobia-regulated responses in *sunn-1* compared to A17 (Table 5-4). Using MapMan (Thimm et al., 2004) biological functional categories indicates that aside from nodulins, the differentially expressed genes showing significant (FC>2 or FC<-2) expression changes were mainly involved in transport and regulation (signaling, regulation of protein activity and TFs) (Figure 5-8 a) in both low and high N.

SUNN-mediated responses were stronger in the presence of rhizobia with a higher number of differentially expressed genes showing strong (FC>2 or FC<-2) expression changes in both low and high N (Table 5-4). Based on the gene abundance in different biological functional categories (Figure 5-8 b) genes involved in regulation (signaling and regulation of protein activity) and stress were more affected by *SUNN*-regulation in the presence of rhizobia at low N than genes with other biological functions. At high N, regulation (signaling and regulation of protein activity) and transport functional categories had the highest number of *SUNN*-regulated genes in the presence of rhizobia (Figure 5-8 b).

These results could suggest that the hypernodulating phenotype and short LRs in *sunn-1* at low N may be mainly the result of fine tuned regulatory cross talk between rhizobia regulated nodulins, TFs, regulation of protein activities and signaling and *SUNN*-regulated signaling, regulation of protein activity and stress responses (Figure 5-9 and 5-11).

5.2.5.1.1 Rhizobia regulated nodulins, putative components of the AON

Differentially expressed nodulins showed significant (FC>2 or FC<-2) expression changes in response to rhizobia and made up for the majority of

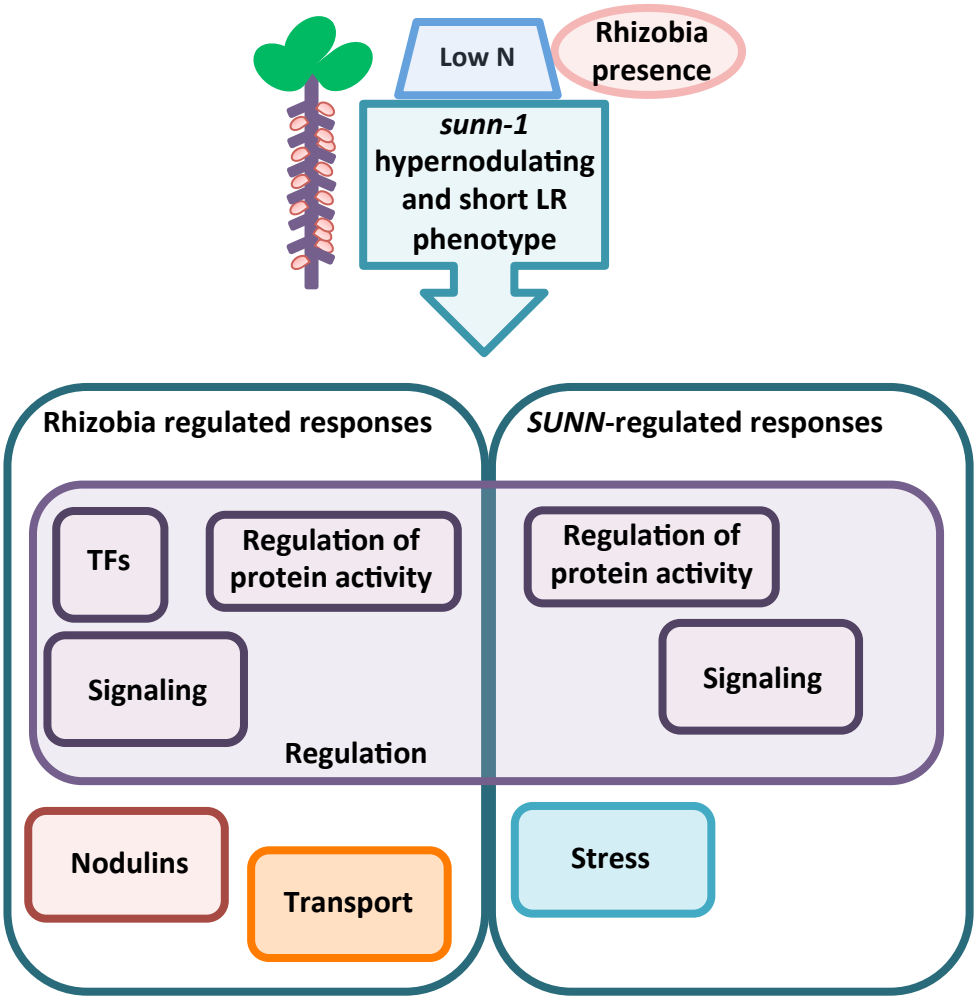


Figure 5-9: The MapMan (Thimm et al., 2004) biological functional categories that based on the Wilcoxon rank sum test ($P < 0.05$) and/or their abundance of significantly (FC>2 or FC<-2) rhizobia-regulated and *SUNN*-regulated transcripts may be the key components of the hypernodulating phenotype with short LRs in *sunn-1* at low N.

the rhizobia-regulated genes at both low and high N in A17 and *sunn-1* (Figure 5-8 a). Aside from two nodulins in *sunn-1* they were all rhizobia-induced at low and high N and mainly belonged to the NCR protein family (as discussed earlier in chapter 4). At low N these genes were more highly expressed in A17 compared to *sunn-1* (Table 5-6).

NCR 172 (Medtr6g045150.1), NCR 181 (Medtr1g011310.1), NCR 122 (Medtr7g071720.2) and Late nodulin (Medtr6g044700.1) are examples of some differentially expressed nodulins (Table 5-3) that had a higher rhizobia-induced expression change in A17 than *sunn-1* at low N. This decrease in the expression level of nodulins in *sunn-1* at low N could be suggestive of their role in AON for controlling the number of nodules. Changes in the expression levels of the *SUNN*-regulated differentially expressed nodulins (FC>2 or FC<-2) in the absence and presence of rhizobia at low N could be also indicative of the role nodulins could have in AON. Some of these differentially expressed nodulins that had the highest rhizobia-regulated expression changes were also significantly (FC>2 or FC<-2) *SUNN*-induced in the absence of rhizobia at low N.

SUNN-regulated expression of the differentially expressed nodulins in low N was found to be dependent on the presence or absence of rhizobia since in -Rhiz a higher number of differentially expressed nodulins showed significant (FC>2 or FC<-2) *SUNN*-regulated responses compared to +Rhiz (Figure 5-8 b). The regulatory effect was also opposite: in -Rhiz 100% of nodulins were *SUNN*-induced but in +Rhiz 86% of the nodulins were *SUNN*-repressed (FC<-2). The differentially expressed nodulins that showed strong (FC>2 or FC<-2) *SUNN*-regulated response at low N were different in -Rhiz and +Rhiz (Table 5-7 a and b). Nodulin 25 (Medtr3g055440), NCR 333 (Medtr4g033900.2) and NCR 172 (Medtr6g045150.1) had the strongest (3<FC<4) *SUNN*-induced response in -Rhiz at low N. Early nodulin-like protein 3 (Medtr5g006040.1), NCR 146 (Medtr1g075500.1) and Nodulin-like protein (Medtr1g099010.1) showed the strongest (-3<FC<-2) *SUNN*-repressed response in +Rhiz at low N. This higher number of *SUNN*-induced

Table 5-6: Rhizobia-regulated nodulins at low and high N in A17 and *sunn-1* (FC>2 or FC<-2). Table is sorted based on descending FC values of the first column and contains some of the NCRs (with the highest expression changes in A17 or *sunn-1* and low or high N) and all other significantly expressed nodulins (FC>2 or FC<-2).

Rhizobia-regulated nodulins		FC			
		Low N		High N	
			sunn		*sunn*
Gene ID	**Annotation**	**A17**	***-1***	**A17**	***-1***
Medtr6g045150.1	NCR 172	8.0	4.1	7.0	7.9
Medtr1g011310.1	NCR 181	7.9	4.5	7.2	8.0
Medtr7g071720.2	NCR 122	7.9	4.6	6.9	7.9
Medtr3g044690.1	NCR 309	7.8	4.3	7.2	7.7
Medtr5g056890.1	NCR 88	7.5	4.2	7.5	7.2
Medtr5g070410.1	NCR 90	7.4	4.3	7.1	8.2
Medtr5g068810.1	NCR 144	7.3	4.8	6.4	7.3
Medtr5g073580.1	NCR 321	7.3	4.3	7.1	6.7
Medtr4g057120.1	NCR 187	7.3	4.6	7.2	7.3
Medtr6g043650.1	NCR 77	7.3	3.7	7.2	6.5
Medtr4g031520.1	NCR 319	7.1	4.7	6.2	7.8
Medtr4g130780.1	Early nodulin	7.0	4.6	5.9	
Medtr6g044700.1	Late nodulin	6.3	3.8	6.2	6.4
Medtr1g042900.1	NCR 56	6.3	5.0	6.0	8.0
Medtr4g031430.1	NCR 318	6.2	4.8	6.4	7.6
Medtr1g030270.3	Early nodule-specific protein	6.0	3.8	5.0	5.3
Medtr1g042900.2	NCR 57	5.9	5.0	5.2	7.8
Medtr6g089330.1	MtN28 protein	5.6	3.3	5.8	5.0
Medtr7g065770.2	Early nodulin ENOD18	4.9	3.6	4.9	5.0
Medtr1g030270.1	Early nodulin	4.6	3.1	4.7	4.1
Medtr7g114890.1	MtN26 protein	4.5	3.3	4.3	5.0
Medtr5g014080.1	NCR 338	4.5	3.2	4.6	4.3
Medtr7g086040.1	MtN20 protein	4.2	4.3	3.7	6.1
Medtr7g114880.1	MtN26 protein	3.9	3.2	3.7	3.6
AC146565 34.1	MtN11 protein	3.5	3.4	2.4	4.9
Medtr6g044570.1	Late nodulin	3.4	2.8	3.5	3.2
Medtr4g026920.1	NCR 324	3.4	2.8	3.0	2.3
Medtr1g030270.2	Early nodule-specific protein	3.3	2.1	3.0	3.6
Medtr4g130800.1	Early nodulin-20	3.0	2.8	2.9	4.8
Medtr2g030470.1	MtN19 protein	2.7	4.6	2.9	5.6
AC146565 18.1	MtN11 protein	2.4	2.2		3.5
AC146565 12.1	MtN11 protein	2.3	2.3	2.2	3.1
Medtr7g016480.1	NCR 339	2.1			
AC146565 19.1	MtN17 protein	2.1	3.2		3.4
Medtr5g032490.1	Nodule-specific glycine-rich protein 1J	2.0			
Medtr1g030220.1	Early nodulin		-2.2		-3.5
Medtr1g099010.1	Nodulin-like protein				-2.1
Medtr2g039230.1	Nodule-specific glycine-rich protein 6A		2.3	2.3	2.7
Medtr5g057910.1	NCR 181			2.2	
Medtr2g044740.1	Nodule-specific glycine-rich protein 5B		2.2		2.3
Medtr5g072280.1	NCR 90		3.5		2.7
Medtr2g045290.1	NCR 318		3.9		3.4

FC -3.5 8.2

Table 5-7: *SUNN*-regulated (FC>2 or FC<-2) nodulins in the (a) absence and (b) presence of rhizobia only at low N and (c) all nodulins affected by *SUNN* in the absence (-Rhiz) or presence (+Rhiz) of rhizobia at high N. Tables contain all *SUNN*-regulated nodulins and are sorted based on descending FC values and in (c) the last column.

(a)

***SUNN*-regulated nodulins in the absence of rhizobia (only at low N)**		
Gene ID	**Annotation**	**FC**
Medtr3g055440.1	Nodulin 25	4.0
Medtr4g033900.2	NCR peptide 333	3.3
Medtr3g055440.2	Nodulin 25	3.3
Medtr6g045150.1	NCR peptide 172	3.0
Medtr1g042900.1	NCR peptide 56	2.9
Medtr1g042910.2	NCR peptide 53	2.7
Medtr7g027180.1	NCR peptide 65	2.6
Medtr1g030270.3	Early nodule-specific protein	2.5
Medtr1g042900.2	NCR peptide 57	2.5
Medtr5g056190.2	NCR peptide 19	2.5
Medtr1g011310.1	NCR peptide 181	2.5
Medtr7g071720.2	NCR peptide 122	2.5
Medtr5g068590.1	NCR peptide 266	2.5
Medtr3g044690.1	NCR peptide 309	2.5
Medtr4g031520.1	NCR peptide 319	2.4
Medtr5g069100.1	NCR peptide 286	2.3
Medtr4g026740.1	NCR peptide 335	2.3
Medtr1g042910.3	NCR peptide 53	2.3
Medtr5g084670.1	Nodule-specific glycine-rich protein 1J	2.2
Medtr5g070410.1	NCR peptide 90	2.2
Medtr3g016020.1	NCR peptide 165	2.2
Medtr6g025480.1	NCR peptide 192	2.2
Medtr4g031430.1	NCR peptide 318	2.1
Medtr3g084910.1	NCR peptide 101	2.0

(b)

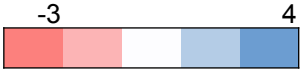
***SUNN*-regulated nodulins in the presence of rhizobia only at low N**		
Gene ID	**Annotation**	**FC**
Medtr4g113820.1	Early nodulin 93 protein	-2.1
Medtr2g083250.1	Early nodulin-like protein 3	-2.1
Medtr5g057580.1	NCR peptide 88	-2.2
Medtr1g099010.1	Nodulin-like protein	-2.3
Medtr1g075500.1	NCR peptide 146	-2.5
Medtr5g006040.1	Early nodulin-like protein 3	-3.0

Table 5-7, continue

(c)

SUNN-regulated nodulins at high N		FC			
		Low N		High N	
Gene ID	Annotation	-Rhiz	+Rhiz	-Rhiz	+Rhiz
Medtr2g030470.1	MtN19 protein		2.8		2.8
Medtr1g030220.1	Early nodulin				-2.3
Medtr2g054210.1	NCR 121			-2.5	
Medtr4g130780.1	Early nodulin	2.7		-2.3	
Medtr4g031380.1	NCR 319	2.7		-2.9	

FC -3 4



nodulins in the absence of rhizobia and the fact that the number of significantly (FC>2 or FC<-2) *SUNN*-regulated nodulins in the presence of rhizobia was around 4 times less and predominantly repressed may indicate that these separate sets of nodulins are affected by rhizobia and could be involved in AON and possibly the rhizobia mediated effect on LR length.

Some differentially expressed NCRs such as NCR 90 (Medtr5g070410.1), NCR 181 (Medtr1g011310.1), NCR 318 (Medtr4g031430.1) that were strongly rhizobia-induced (4<FC<8) in both genotypes at low and high N, did not show a huge variation in their expression levels between low and high N in A17. However there was about a two times increase in their expression level at high N compared to low N in *sunn-1.*

There was no variation in the expression changes of some nodulins such as MtN20 protein (Medtr7g086040.1) and MtN11 protein (AC146565_34.1) between A17 and *sunn-1* in response to rhizobia at low N but at high N their expression level was about 2 times higher in *sunn-1* than A17 (Table 5-3). Such variations in the rhizobia-regulated expression levels of these nodulins between A17 and *sunn-1* at low and high N may suggest that they may be involved in controlling the number of nodules according to the plant N status.

5.2.5.1.2 Putative Regulators of the AON

TFs play a critical role in different biological pathways by negative or positive regulation of the expression of relevant genes. The number of rhizobia-regulated (FC>2 or FC<-2) differentially expressed TFs in *sunn-1* was around three times higher than A17 at low N. This number was also higher in *sunn-1* at high N however a higher percentage of TFs were induced in A17 (Figure 5-8 a).

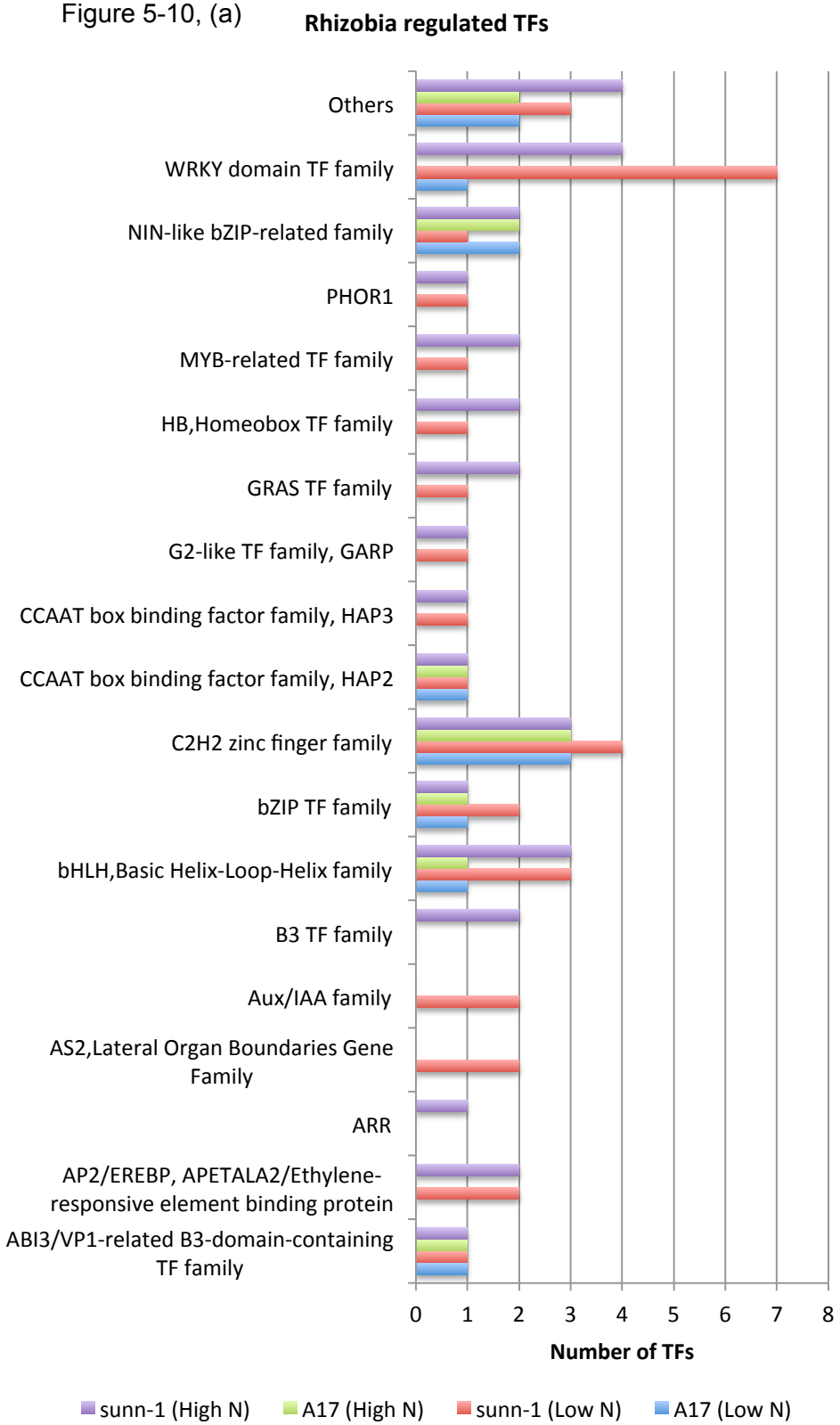
A higher percentage of the differentially expressed TFs showed strong (FC<-2) rhizobia-repressed response at low N in *sunn-1* (43%) than A17 (only 8%). These TFs may be involved in regulation of the expression of AON

components at low N in nodulating plants and regulators of LR and nodule balance. At high N the number of rhizobia-induced TFs was greater, particularly in A17 where 100% of the TFs were upregulated (compared to 76% upregulated in *sunn-1*). The *SUNN*-regulated effect on the expression of differentially expressed TFs at low N was rhizobia dependent and only in the presence of rhizobia they showed significant (FC>2 or FC<-2) expression changes (Figure 5-8 b and 5-10 b).

A higher number of differentially expressed WRKY TFs were affected by the rhizobia-regulated response at low N and also high N (FC>2 or FC<-2) than differentially expressed TFs from other families in *sunn-1* (Figure 5-10 a). These WRKY TFs were significantly (FC>2 or FC<-2) differentially expressed at low and high N only in *sunn-1* except for Medtr2g075610.1 that was also rhizobia-suppressed in A17 (FC=-2.2). Aside from WRKY 33 (FC=2.8) all these TFs were strongly (FC<-2) rhizobia-suppressed at low N. Members of the WRKY TF family act as activators and repressors in controlling several biological processes including development, immunity and stress responses (Rushton et al., 2010, Canales et al., 2014). The rhizobia-regulated response of the significantly expressed WRKY TFs could suggest that they may be among the key regulators involved in AON.

Four differentially expressed WRKY TFs including WRKY 7 and 22 along with some other differentially expressed TFs from TF families involved in plant growth, development and nodulation (Andriankaja et al., 2007, Carretero-Paulet et al., 2010, Godiard et al., 2011) such as bHLH 85 (Medtr5g005110.1) and Ethylene-responsive TF (ERF) ERF026 (Medtr1g101550.1) from the AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family were rhizobia-suppressed only at low N (Table 5-8). These TF families are among TFs involved in nodulation and the cross talk with the components of the signaling pathways to coordinate different steps of the nodulation pathway, from rhizobial infection to nodule organogenesis. The differentially expression of these TFs as rhizobia-suppression only at low N could make them candidates involved in

Figure 5-10, (a)


Rhizobia regulated TFs
Others
WRKY domain TF family
NIN-like bZIP-related family
PHOR1
MYB-related TF family
HB,Homeobox TF family
GRAS TF family
G2-like TF family, GARP
CCAAT box binding factor family, HAP3
CCAAT box binding factor family, HAP2
C2H2 zinc finger family
bZIP TF family
bHLH,Basic Helix-Loop-Helix family
B3 TF family
Aux/IAA family
AS2,Lateral Organ Boundaries Gene Family
ARR
AP2/EREBP, APETALA2/Ethylene-responsive element binding protein
ABI3/VP1-related B3-domain-containing TF family
0
1
2
3
4
5
6
7
8
Number of TFs
sunn-1 (High N)
A17 (High N)
sunn-1 (Low N)
A17 (Low N)

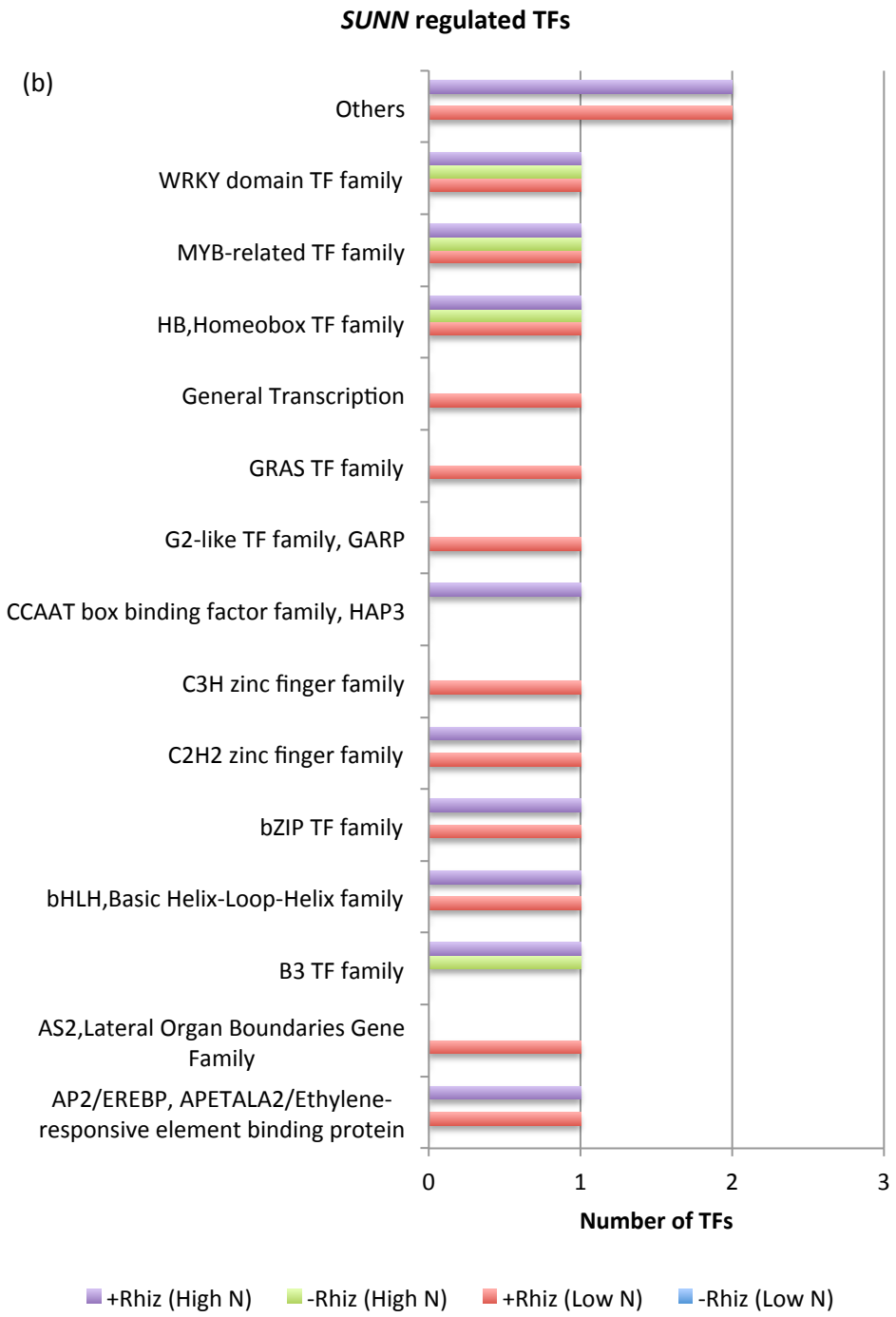


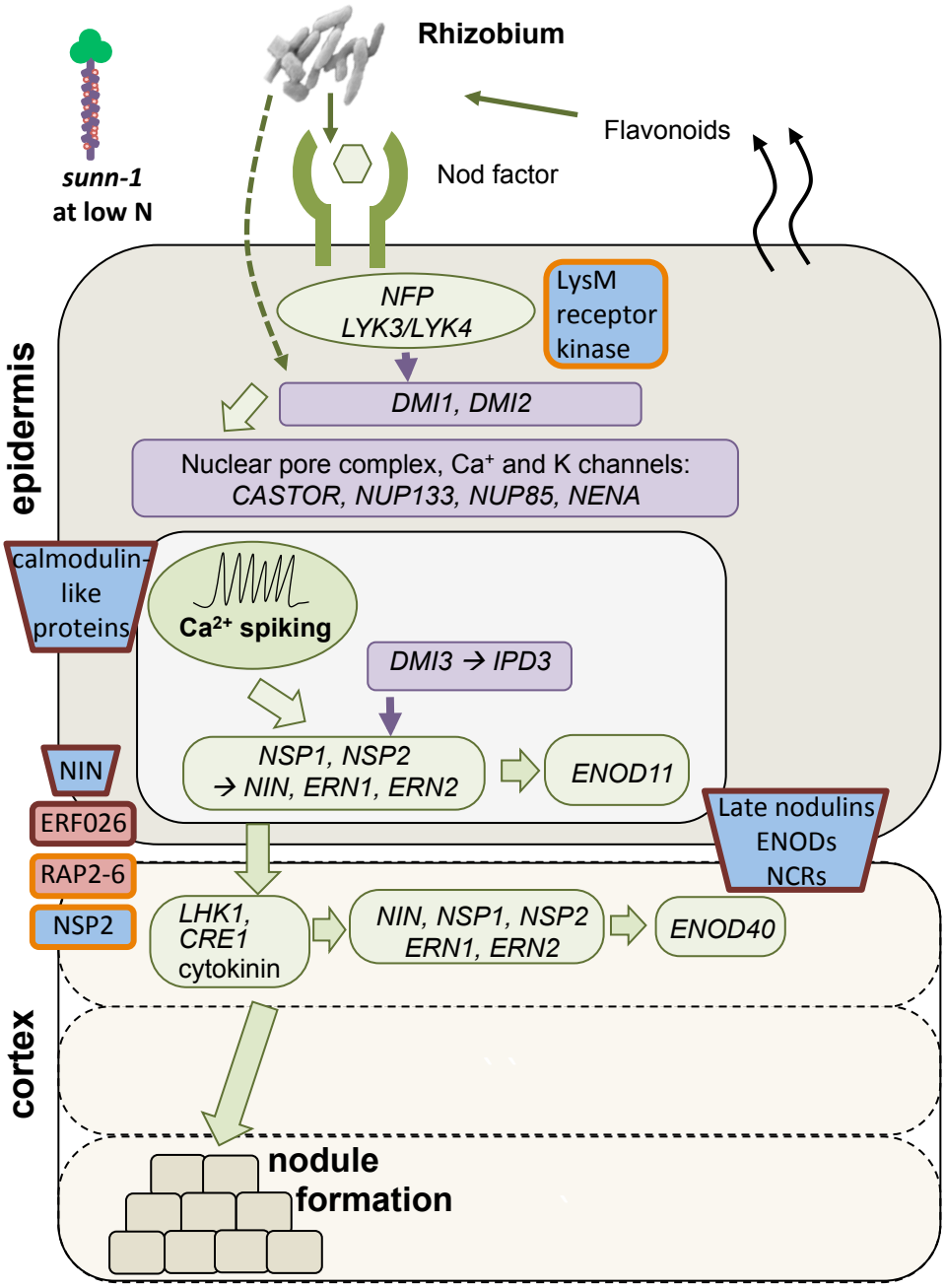
Figure 5-10: TF families identified by MapMan software (Thimm et al., 2004) that may have a putative role in AON and the balance between nodule number and LR development because of their significant (FC>2 or FC<-2) expression changes in response to rhizobia and/or *SUNN*. (a) Rhizobia responsive TFs in A17 and *sunn-1*, and (b) *SUNN* responsive TFs in the absence (-Rhiz) or presence (+Rhiz) of rhizobia at low or high N. The horizontal axis shows the number of TFs in each family.

transcriptional regulation during nodule and LR development in response to low N with a putative role in AON.

The differentially expressed MYB TF (Medtr4g108430.1), MYB family TF-like protein (Medtr7g010210.1), WRKY 33 (FC=3.4), WRKY 73 (FC=2.2) and ERF 12 (Medtr7g085810.1) with FC=2.6 were among the significantly rhizobia-induced TFs only in *sunn-1* at high N. The MYB domain TF family are key regulators in networks controlling development, metabolism and responses to biotic and abiotic stresses (Dubos et al., 2010). Considering the ability of *sunn-1* to nodulate at high N and the regulatory roles of these TF families in development and response to biotic and abiotic stress these TFs could be candidates involved in controlling nodule formation according to N availability. This could be especially true for WRKY 33 and ERF 12. ERF 12 was also significantly *SUNN*-induced (FC=2.5) only at high N in the presence of rhizobia and the *SUNN*-regulated response of WRKY 33 was rhizobia dependent, it was significantly induced (FC>3) at both low and high N only in the presence of rhizobia. These rhizobia dependent responses of ERF 12 and WRKY 33 at high N (rhizobia-induced in *sunn-1* and *SUNN*-induced in the presence of rhizobia) could make them putative regulators of nodule formation according to the N status.

In this study some of the components of the nodulation-signaling pathway that showed significant expression changes in response to rhizobia and *SUNN* at low N were identified (Figure 5-11). Because of their significant rhizobia or *SUNN*-regulated expression changes (as explained below) it was proposed that they might have putative roles in the AON (Figure 5-11).

The differentially expressed LysM receptor kinase (Medtr5g086130.1) was *SUNN*-induced (FC=2) at low N in the presence of rhizobia. The differentially expressed calmodulin-like proteins 1, 4 and 5 (Medtr3g055570.1, Medtr3g055490.1, Medtr3g055510.1 respectively) were rhizobia-induced (2.3 < FC < 6.8) at low and high N in both A17 and *sunn-1* showing variation in their expression patterns between genotypes and different treatments (Table



SUNN-induced at low N and +Rhiz

SUNN-repressed at low N and +Rhiz

Rhizobia-repressed at low N in *sunn-1*

Rhizobia-induced at low N, lower expression level in *sunn-1*

Figure 5-11: A model for the main steps of nodulation signaling pathway. Proposed TFs, signaling genes and nodulins involved in the nodulation pathway with a putative role in AON are added to the model. These are chosen because of their significant (FC>2 or FC<-2) rhizobia-regulated response in *sunn-1* or *SUNN*-regulated response in the presence of rhizobia (+Rhiz) at low N. The transcripts are shown as rectangles with orange (*SUNN*-regulated in the presence of rhizobia) or red (rhizobia-regulated at low N in *sunn-1*) lines and trapezoids (rhizobia-induced with a lower expression level in *sunn-1* compared to A17) filled with blue (induced) or red (repressed) color.

Table 5-8: A selection of rhizobia-regulated (FC>2 or FC<-2) genes at low and/or high N in A17 and/or *sunn-1* assigned to different functional categories using MapMan software (Thimm et al., 2004). Tables are sorted alphabetically by the functional categories column.

Rhizobia-regulated				FC			
				Low N		High N	
	Functional categories	Gene ID	Annotation	A17	*sunn -1*	A17	*sunn -1*
Transcriptional regulators	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr1g101550.1	Ethylene-responsive TF ERF026		-2.2		
	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr7g085810.1	Ethylene-responsive TF 12				2.6
	AS2,Lateral Organ Boundaries Gene Family	Medtr3g031660.1	LOB domain-containing protein 12		-2.1		
	AS2,Lateral Organ Boundaries Gene Family	Medtr5g083960.1	LOB domain-containing protein 15		-2.0		
	bHLH,Basic Helix-Loop-Helix family	Medtr4g009000.1	TF bHLH25				-2.3
	bHLH,Basic Helix-Loop-Helix family	Medtr4g098210.1	TF bHLH25		3.4		3.9
	bHLH,Basic Helix-Loop-Helix family	Medtr5g005110.1	TF bHLH85		-2.2		
	MYB domain transcription factor family	Medtr4g108430.1	MYB transcription factor				2.0
	MYB domain transcription factor family	Medtr7g010210.1	MYB family transcription factor-like protein				2.7
	MYB-related transcription factor family	Medtr3g104370.1	MYB transcription factor		-2.1		
	NIN-like bZIP-related family	Medtr4g068000.1	Nodule inception protein (NIN)	3.1		3.2	2.9
	WRKY domain TF family	Medtr1g015140.1	WRKY TF 7		-2.2		
	WRKY domain TF family	Medtr2g075610.1	WRKY TF	-2.2	-3.1		-2.1
	WRKY domain TF family	Medtr2g075680.1	WRKY TF		-2.3		
	WRKY domain TF family	Medtr2g075700.1	WRKY TF		-2.3		
	WRKY domain TF family	Medtr4g122530.1	WRKY TF 33		2.8		3.4
	WRKY domain TF family	Medtr5g067700.1	WRKY TF		-2.2		-2.4
	WRKY domain TF family	Medtr7g009730.1	WRKY TF 73				2.2
	WRKY domain TF family	Medtr8g005750.1	WRKY TF 22		-2.4		

Table 5-8, continue

	Rhizobia-regulated			FC			
				Low N		High N	
					sunn		*sunn*
	Functional categories	Gene ID	Annotation	A17	*-1*	A17	*-1*
Signaling	calcium	Medtr3g055490.1	Calmodulin-like protein 4	4.1	2.3	5.4	4.6
	calcium	Medtr3g055510.1	Calmodulin-like protein 5	6.1	4.1	6.0	6.4
	calcium	Medtr3g055570.1	Calmodulin-like protein 1	6.7	3.9	5.8	6.8
	receptor kinases.DUF 26	AC235006_23.1	Cysteine-rich receptor-like protein kinase 25		-2.5		-2.6
	receptor kinases.DUF 26	AC235009_43.1	Cysteine-rich receptor-like protein kinase 29				2.2
	receptor kinases.DUF 26	Medtr1g031450.1	Kinase-like protein				-2.3
	receptor kinases.DUF 26	Medtr1g073320.1	Cysteine-rich repeat secretory protein 3				2.0
	receptor kinases.DUF 26	Medtr4g126930.1	Cysteine-rich receptor-like protein kinase 41	2.3	4.3	2.5	5.5
	receptor kinases.DUF 26	Medtr6g091230.1	Brassinosteroid LRR receptor kinase				-2.1
	receptor kinases.leucine rich repeat XI	Medtr4g070950.1	CLV1-like receptor kinase		-2.0		-2.5
	receptor kinases.leucine rich repeat XI	Medtr4g070970.1	Receptor protein kinase CLAVATA1				-2.1
	receptor kinases.leucine rich repeat XI	AC159090_21.1	Cysteine-rich receptor-like protein kinase 41		-2.3		
	receptor kinases.leucine rich repeat XI	Medtr5g019070.1	LRR receptor-like serine/threonine-protein kinase FEI 1		4.3		4.3
	receptor kinases.leucine rich repeat XI	Medtr5g025840.1	Kinase-like protein		3.2		3.1
	receptor kinases.leucine rich repeat XI	Medtr5g025850.1	Receptor-like protein kinase	2.4	4.2	3.5	5.2
	receptor kinases.lysine motif	Medtr5g086110.1	LysM domain-containing receptor-like kinase 4		2.6		3.2
	receptor kinases.misc	Medtr4g090240.1	Stress-induced receptor-like kinase 2		2.2		3.3
	receptor kinases.misc	Medtr7g071790.1	Wound-induced protein 1	2.4		2.3	

Table 5-8, continue

	Rhizobia-regulated			FC			
				Low N		High N	
					sunn		*sunn*
	Functional categories	Gene ID	Annotation	A17	*-1*	A17	*-1*
Regulation of protein activity	protein.degradation.aspartate protease	Medtr5g044770.1	Aspartic proteinase nepenthesin-1			2.0	2.3
	protein.degradation.cysteine protease	AC233675_19.1	Cysteine protease 5	7.0		6.5	7.5
	protein.degradation.cysteine protease	Medtr2g083930.1	Cysteine proteinase				-2.4
	protein.degradation.cysteine protease	Medtr3g044270.1	Cysteine proteinase	4.2		4.2	4.2
	protein.degradation.cysteine protease	Medtr4g080700.1	Cysteine proteinase	2.0		2.0	
	protein.degradation.cysteine protease	Medtr4g080730.1	Cysteine proteinase			2.5	2.2
	protein.degradation.metalloprotease	Medtr5g036110.1	MtN9 protein	3.1		3.3	3.5
	protein.degradation.ubiquitin.E3.RING	Medtr4g106680.1	RING finger protein				-2.1
	protein.degradation.ubiquitin.E3.RING	Medtr5g064980.1	RING finger-like	4.5	3.8	4.2	5.0
	protein.postranslational modification	Medtr1g098300.1	Cell division protein kinase 9		2.9		3.2
	protein.postranslational modification	Medtr5g069000.1	Microtubule-associated serine/threonine-protein kinase 2	5.6	3.9	4.9	4.9
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VI	Medtr7g113980.1	Cysteine-rich receptor-like protein kinase 18		2.6		2.3
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr4g061990.1	Protein kinase-like protein				2.7
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr4g062030.1	Receptor protein kinase-like protein				2.5
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr4g129010.1	Somatic embryogenesis receptor kinase 2		2.2		2.2
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr5g035030.1	Protein kinase family protein		2.5		2.6
	protein.targeting.secretory pathway.unspecified	Medtr8g039990.1	Mitochondrial inner membrane protease subunit 1		3.5		3.1

Table 5-8, continue

Rhizobia-regulated				FC			
				Low N		High N	
					sunn		*sunn*
	Functional categories	Gene ID	Annotation	A17	*-1*	A17	*-1*
Hormone metabolism	abscisic acid.synthesis-degradation	Medtr3g109610.2	Dioxygenase RAMOSUS1		2.1		
	auxin.induced-regulated-responsive-activated	Medtr2g081860.1	Indole-3-acetic acid-amido synthetase GH3.6				2.2
	auxin.induced-regulated-responsive-activated	Medtr4g072980.1	Auxin-induced SAUR-like protein	3.1	2.9		
	auxin.induced-regulated-responsive-activated	Medtr4g072980.1	Auxin-induced SAUR-like protein			4.0	4.2
	auxin.induced-regulated-responsive-activated	Medtr5g016320.1	Indole-3-acetic acid-amido synthetase GH3.3		2.5		2.0
	cytokinin.signal transduction	Medtr2g100880.1	Histidine phosphotransfer protein				-3.5
	cytokinin.synthesis-degradation	Medtr2g039410.1	Cytokinin dehydrogenase 3		2.4		
	cytokinin.synthesis-degradation	Medtr3g036100.1	Cytokinin dehydrogenase 1				-2.4
	ethylene.synthesis-degradation	Medtr2g068960.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1				2.9
	ethylene.synthesis-degradation	Medtr7g045650.1	Protein SRG1		-2.5		
	gibberelin.induced-regulated-responsive-activated	AC235748_1016.1	Snakin-1			2.5	
	gibberelin.induced-regulated-responsive-activated	Medtr1g018640.1	Snakin-1		-2.4		-2.5
	gibberelin.synthesis-degradation.ent-kaurenoic acid hydroxylase/oxygenase	Medtr5g014250.1	Cytochrome P450 ent-kaurenoic acid oxidase		2.1		2.4
	jasmonate.synthesis-degradation.12-Oxo-PDA-reductase	Medtr5g008040.1	12-oxophytodienoate reductase	3.4	2.4	3.3	2.7
	jasmonate.synthesis-degradation.lipoxygenase	AC233656_18.1	Lipoxygenase				2.8
	jasmonate.synthesis-degradation.lipoxygenase	Medtr8g018650.1	Lipoxygenase				-2.0
	salicylic acid.synthesis-degradation	Medtr3g052700.1	S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase-like protein				2.3
	salicylic acid.synthesis-degradation	Medtr3g117060.1	Salicylic acid/benzoic acid carboxyl methyltransferase		-2.9		-2.7
	salicylic acid.synthesis-degradation	Medtr7g080940.1	Uncharacterized UDP-glucosyltransferase At1g05670	4.2	2.8	4.9	5.0

Table 5-8, continue

Functional categories	Rhizobia-regulated Gene ID	Annotation	FC Low N A17	FC Low N *sunn -1*	FC High N A17	FC High N *sunn -1*
CLAVATA like (CLE) peptides	Medtr6g009390	MtCLE2	0.02	-1.2	0.5	-1.0
	Medtr5g014860	MtCLE4	1.3	1.5	1.4	1.2
	Medtr7g058790	MtCLE6	-0.005	-0.5	0.03	-0.5
	Medtr7g089320	MtCLE7	0.1	-0.8	-0.1	-1.1
	Medtr5g089080	MtCLE21	-0.2	-0.9	0.1	-0.8
	Medtr4g070970.1	Receptor protein kinase CLAVATA1	-0.1	-1.5	-0.4	-2.1
	Medtr4g070950.1	CLV1-like receptor kinase	-0.3	-2.0	-0.4	-2.5
CEP peptides	Medtr5g017710	MtCEP5	-0.6	-1.1	0.3	-1.1
	AC233112_1014	MtCEP8	-0.4	-0.6	0.5	-0.4
	Medtr8g072170	MtCEP11	0.5	-0.8	-0.4	-0.4

FC -3.5 7.5

Table 5-9: A selection of SUNN-regulated (FC>2 or FC<-2) genes at low and/or high N in the presence (+Rhiz) or absence (-Rhiz) of rhizobia assigned to different functional categories using MapMan software (Thimm et al., 2004). Tables in each functional category are sorted based on descending FC values of the first column. Tables are sorted alphabetically by the functional categories column.

	SUNN-regulated			Low N		High N	
	Functional categories	Gene ID	Annotation	-Rhiz	+Rhiz	-Rhiz	+Rhiz
Transcriptional regulators	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr3g098580.1	Ethylene-responsive TF RAP2-6		-2.4		
	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	Medtr7g085810.1	Ethylene-responsive TF 12				2.5
	bHLH,Basic Helix-Loop-Helix family	Medtr4g098210.1	TF bHLH25		2.6		
	bHLH,Basic Helix-Loop-Helix family	Medtr4g098210.1	TF bHLH25				2.2
	C3H zinc finger family	AC235678_19.1	Zinc finger CCCH domain-containing protein 15		-2.7		
	GRAS transcription factor family	Medtr3g072710.1	Nodulation-signaling pathway 2 protein		2.2		
	RNA.regulation of transcription.MYB-related transcription factor family	Medtr3g104370.1	MYB transcription factor		-3.7	-2.1	-2.6
	WRKY domain transcription factor family	Medtr4g122530.1	WRKY TF 33		3.5		3.0
	WRKY domain transcription factor family	Medtr5g067700.1	WRKY TF			2.0	
Signaling	receptor kinases.leucine rich repeat XI	Medtr4g070950.1	CLV1-like receptor kinase		-2.6		-2.7
	receptor kinases.leucine rich repeat XI	Medtr4g070970.1	Receptor protein kinase CLAVATA1		-3.1	-2.0	-3.7
	receptor kinases.lysine motif	Medtr5g086110.1	LysM domain-containing receptor-like kinase 4		3.5		3.3
	receptor kinases.lysine motif	Medtr5g086130.1	LysM receptor kinase 3		2.0		
	signalling.receptor kinases.DUF 26	Medtr4g091770.1	Cysteine-rich receptor-like protein kinase 6		-2.1		
	signalling.receptor kinases.DUF 26	Medtr4g091780.1	Serine/threonine kinase receptor		-2.3		
	signalling.receptor kinases.DUF 26	Medtr4g091780.1	Serine/threonine kinase receptor				-2.7
	signalling.receptor kinases.DUF 26	Medtr4g126930.141	Cysteine-rich receptor-like protein kinase		2.6		2.7
	signalling.receptor kinases.leucine rich repeat III	Medtr4g014350.1	Leucine-rich repeat receptor-like protein kinase		-2.5		
	signalling.receptor kinases.leucine rich repeat IX	Medtr2g087230.1	Receptor-like protein kinase		-2.2		
	signalling.receptor kinases.leucine rich repeat XI	Medtr4g070950.1	CLV1-like receptor kinase		-2.6		-2.7
	signalling.receptor kinases.leucine rich repeat XI	Medtr4g070970.1	Receptor protein kinase CLAVATA1		-3.1	-2.0	-3.7

Table 5-9, continue

	SUNN-regulated Functional categories	Gene ID	Annotation	Low N -Rhiz	Low N +Rhiz	High N -Rhiz	High N +Rhiz
Signaling	signalling.receptor kinases.leucine rich repeat XI	Medtr5g025850.1	Receptor-like protein kinase		2.5		2.2
	signalling.receptor kinases.leucine rich repeat XI	Medtr6g065220.1	Receptor protein kinase				2.4
	signalling.receptor kinases.lysine motif	Medtr5g086110.1	LysM domain-containing receptor-like kinase 4		3.5		3.3
	signalling.receptor kinases.lysine motif	Medtr5g086130.1	LysM receptor kinase 3		2.0		
	signalling.receptor kinases.lysine motif	Medtr5g086330.1	Wall-associated receptor kinase-like 22				2.3
	signalling.receptor kinases.misc	Medtr3g031270.1	Wound-induced protein 1		2.2		
	signalling.receptor kinases.misc	Medtr4g090240.1	Stress-induced receptor-like kinase 2		2.1		
	signalling.receptor kinases.misc	Medtr5g078030.1	Cysteine-rich repeat secretory protein 60		-2.1		
Regulation of protein activity	protein.aa activation.asparagine-tRNA ligase	Medtr8g011530.1	Asparaginyl-tRNA synthetase		-2.0		
	protein.degradation.aspartate protease	Medtr1g093100.1	Xyloglucan-specific endoglucanase inhibitor protein		-2.3		
	protein.degradation.aspartate protease	Medtr3g096930.1	Aspartic proteinase Asp1		-2.0		
	protein.degradation.cysteine protease	AC233675_19.1	Cysteine protease 5	2.498			
	protein.degradation.cysteine protease	Medtr5g022560.1	Cysteine proteinase	2.375			
	protein.degradation.ubiquitin.E3.RING	Medtr3g088100.1	RING finger protein 38		-2.3		
	protein.degradation.ubiquitin.E3.RING	Medtr4g057310.1	RING finger and CHY zinc finger domain-containing protein 1		2.3		3.0
	protein.degradation.ubiquitin.E3.RING	Medtr5g082940.1	RING finger protein 43		2.3		
	protein.postranslational modification	Medtr2g011200.1	Cysteine-rich receptor-like protein kinase 10		-2.1		
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VI	Medtr7g113980.1	Cysteine-rich receptor-like protein kinase 18		2.0		2.1
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr2g087230.1	Receptor-like protein kinase		-2.2		
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr4g061420.1	Protein kinase-like protein				2.7
	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VII	Medtr5g086330.1	Wall-associated receptor kinase-like 22				2.3

Table 5-9, continue

	SUNN-regulated Functional categories	Gene ID	Annotation	Low N -Rhiz	Low N +Rhiz	High N -Rhiz	High N +Rhiz
Hormone metabolism	abscisic acid.induced-regulated-responsive-activated	Medtr5g091800.1	HVA22-like protein f				-2.4
	abscisic acid.synthesis-degradation	Medtr3g109610.2	Dioxygenase RAMOSUS1				2.4
	abscisic acid.synthesis-degradation	Medtr7g045370.1	Carotenoid cleavage dioxygenase 7, chloroplastic		-3.0		
	auxin.induced-regulated-responsive-activated	Medtr5g091070.1	SAUR1-auxin-responsive SAUR family member		-2.0		-2.4
	auxin.induced-regulated-responsive-activated	Medtr5g093520.1	Auxin-induced in root cultures protein 12		-2.4		
	auxin.induced-regulated-responsive-activated	Medtr5g099010.1	Auxin-independent growth promoter		-2.3		
	auxin.signal transduction	Medtr7g079720.1	Auxin Efflux Carrier		-2.0		-2.4
	cytokinin.signal transduction	Medtr2g100880.1	Histidine phosphotransfer protein		-2.1		-2.6
	cytokinin.synthesis-degradation	Medtr4g044110.1	Cytokinin oxidase				-2.0
	ethylene.synthesis-degradation	Medtr2g068960.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1				2.4
	ethylene.synthesis-degradation	Medtr2g069020.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1		2.4		
	ethylene.synthesis-degradation	Medtr4g093840.1	Flavonol synthase/flavanone 3-hydroxylase		2.2		3.2
	ethylene.synthesis-degradation	Medtr7g045650.1	Protein SRG1		-2.9		
	ethylene.synthesis-degradation	Medtr8g009160.1	1-aminocyclopropane-1-carboxylate oxidase homolog 4				2.1
	gibberelin.induced-regulated-responsive-activated	Medtr1g018640.1	Snakin-1		-2.8		-2.6
	gibberelin.induced-regulated-responsive-activated	Medtr5g078220.1	GASA5-like protein		-2.2		-2.5
	gibberelin.synthesis-degradation	Medtr7g090520.2	Gibberellin 20 oxidase 2		-2.0		
	salicylic acid.synthesis-degradation	Medtr3g117060.1	Salicylic acid/benzoic acid carboxyl methyltransferase		-3.6		-2.7

Table 5-9, continue

SUNN-regulated				Low N		High N	
	Functional categories	Gene ID	Annotation	-Rhiz	+Rhiz	-Rhiz	+Rhiz
Stress	stress.abiotic.unspecified	Medtr1g016480.1	Abscisic acid receptor PYL2		-2.5		
	stress.abiotic.unspecified	Medtr2g035120.1	Pathogenesis-related protein PR10		-2.1		
	stress.abiotic.unspecified	Medtr4g010340.1	Germin-like protein 9		-2.9		-3.4
	stress.biotic	Medtr3g031750.1	Cc-nbs-lrr resistance protein		2.3		
	stress.biotic	Medtr6g046750.1	Cc-nbs-lrr resistance protein		-2.4		
	stress.biotic	Medtr7g116850.1	Acidic endochitinase			2.1	
	stress.biotic.PR-proteins	AC159090_9.1	TIR-NBS disease resistance-like protein		-2.3		
	stress.biotic.PR-proteins	Medtr2g083650.1	Disease-resistance protein		2.2		
	stress.biotic.PR-proteins	Medtr3g079780.1	Tir-nbs-lrr resistance protein		2.4		2.0
	stress.biotic.PR-proteins	Medtr5g063740.1	Receptor-like kinase-like		-2.0		
	stress.biotic.PR-proteins	Medtr5g092340.1	Disease resistance-like protein GS0-1		-2.3		
	stress.biotic.PR-proteins	Medtr7g093820.1	Disease resistance response protein 206		-2.2		
	stress.biotic.PR-proteins.proteinase inhibitors.trypsin inhibitor	Medtr6g078250.1	Pathogen-inducible trypsin-inhibitor-like protein		-3.5		-2.4
	stress.biotic.PR-proteins.proteinase inhibitors.trypsin inhibitor	Medtr6g078280.1	Miraculin		-3.0		
CLAVATA like (CLE) peptides		Medtr6g009390	MtCLE2	-0.6	-1.9	-1.6	-0.1
		Medtr5g014860	MtCLE4	0.3	0.5	0.7	0.9
		Medtr7g058790	MtCLE6	-0.4	-0.9	-0.5	0.002
		Medtr7g089320	MtCLE7	0.03	-1.0	-0.9	0.1
		Medtr5g089080	MtCLE21	-0.3	-0.9	-1.1	-0.2
		Medtr4g070970.1	Receptor protein kinase CLAVATA1	-1.7	-3.1	-3.7	-2.0
		Medtr4g070950.1	CLV1-like receptor kinase	-0.8	-2.6	-2.7	-0.7
CEP peptides		Medtr5g017710	MtCEP5	-0.6	-1.1	-0.6	0.8
		AC233112_1014	MtCEP8	-1.1	-1.3	-0.9	0.004
		Medtr8g072170	MtCEP11	-0.6	-1.9	-0.2	-0.3

FC -3.7 3.5

5-8). However, at low N the expression levels of these genes were almost two times lower in *sunn-1* than A17. The lower expression levels of the significantly induced calmodulin proteins at low in *sunn-1* that shows the hyper nodulating phenotype and the rhizobia dependent *SUNN*-induced expression of LysM receptor kinase could suggest that these could be involved in the regulation of nodule numbers. In the nodulation signaling pathway, LysM receptor complexes perceive Nod factor (Amor et al., 2003). LysM activation triggers a signaling cascade that induces nucleus calcium spiking leading to the activation of DMI3. DMI3 activates the nodulation TFs NSP2, NIN and ERN (member of the AP2/ERF family). These TFs regulate the expression of early nodulins (ENODs) such as ENOD11 (Oldroyd, 2013) (Figure 5-11).

Of the differentially expressed TFs involved in the nodulation-signaling pathway NIN (Medtr4g068000.1), showed significant (FC>2) rhizobia-induction at low and high N in A17 and only at high N in *sunn-1.* Changes in the expression level of NIN were almost the same (FC=3) in A17 at low and high N and *sunn-1* at high N (Table 5-8). NIN is a key regulator of the nodulation pathway with a potential negative role in transcriptional regulation of *ENOD11* (Andriankaja et al., 2007, Marsh et al., 2007). Recent studies on *L. japonicus* have provided evidence suggesting that this bifunctional TF negatively regulates infection but positively regulates nodule organogenesis (Yoro et al., 2014). The lower expression level of NIN only in *sunn-1* at low N where it shows the hypernodulating phenotype could be indicative of the role this TF may have in AON.

Of the differentially expressed ERF TFs ERF026 (Medtr1g101550.1) was rhizobia-repressed (FC=-2.2) at low N in *sunn-1* and RAP2-6 (Medtr3g098580.1) was *SUNN*-repressed (FC=2.4) in the presence of rhizobia. AtRAP2-6 participates in plant developmental processes as well as biotic and/or abiotic stress signaling such as ABA, salt and osmotic stress responses (Zhu et al., 2010, Krishnaswamy et al., 2011). ERFs are part of the AP2/EREBP TF family and are involved in signal transduction and regulation

of defence or regulation genes in response to abiotic and biotic stress such as pathogen and defence responses (Xu et al., 2011). Members of this family such as ERN (ERF for required nodulation) and EFD (ethylene response factor required for nodule differentiation) are key regulators of the nodulation pathway including controlling nodule number and differentiation (Andriankaja et al., 2007, Vernié et al., 2008). The differentially expressed Nodulation-signaling pathway 2 protein (NSP2), Medtr3g072710.1, was *SUNN*-induced (FC=2.2) only at low N in the presence of rhizobia. NSP2 together with NSP1 act as positive regulators of ERN1 and ENOD11 transcription (Cerri et al., 2012). Thus this co-*SUNN* induction of ERF RAP2-6 and NSP2 at low N and in the presence of rhizobia could be indicative of their role in regulating the ENODs and other nodulins of the nodulation pathway.

5.2.5.1.3 Stress related genes with putative role in AON

Based on the number of significantly differentially expressed (FC>2 or FC<-2) expressed genes in the MapMan's stress functional category, the stress response was stronger in *SUNN*-regulated genes at low N and in the presence of rhizobia. The stress response was rhizobia dependent and also affected by N concentration because at low N they were significantly differentially expressed (FC>2 or FC<-2) expressed only in +Rhiz and their number was 4 times more at low N than high N. According to MapMan functional category, 62% were related to biotic stress (Table 5-9). The majority (84%) of these stress related genes were *SUNN*-repressed. From all these it could be suggested that these stress related genes (Table 5-9) could be involved in rhizobia-legume symbiotic pathways and control of nodule formation.

5.2.6 Analysis of differentially expressed gene promoters identifies putative regulatory motifs mediating *SUNN* responses

As discussed above, the identified genes that were differentially expressed (FC>2 or FC<-2) during nodulation and under low and high N concentrations in A17 and *sunn-1* are proposed to have a role in AON and

balance between LR and nodule number according to the plant N status. To have a better understanding of the transcriptional regulation of these genes, promoters of genes for transcriptional motifs were studied.

As explained earlier, differentially expressed genes were assigned to 12 different '*groups'* (Table 5-3) representing A17 and *sunn-1* responses to low or high N concentrations in the presence or absence of rhizobia, rhizobia at low or high N and *SUNN*-regulation at low or high N in the presence or absence of rhizobia. In each group, motifs analysis was carried out by using the MEME Launcher and Browser tool MEME-LaB (Brown et al., 2013) to search for sequence patterns that occurred repeatedly (i.e. motifs) in the co-expressed genes belonging to the group. The maximum length of promoter sequence to be searched were specified as either 200, 500 and 1000 bp that was measured from the transcription start site upstream in the 5' direction. The analysis was run using 9 different datasets which contained the 12 groups of differentially expressed genes as defined in Table 5-3 with different FC cutoffs (Table 5-10) and for each dataset with 3 different promoter maximum lengths (200, 500 and 1000 bp) = 27 runs. This allowed analysis of the data as: 1) genes showing the strongest response (datasets 1-3) that were genes with FC>3 or FC<-3 (where the highest FC value was 2 or -2 this cutoff was used instead); 2) genes with FC>2 or FC<-2 that were also studied in different MapMan functional categories (data sets 4-6); 3) all genes without identifying a FC cutoff (data sets 7-9); 4) data sets containing both over expressed and under expressed genes (data sets 1, 4 and 7) and data sets with only over or under expressed genes (data sets 8 and 9).

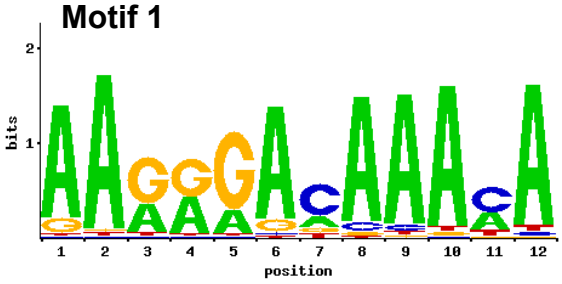
To specify the most significant motifs these results were first filtered using the following criteria: %Promoters ≥ 20 (to identify the most common motifs), Info content ≥ 10 (to choose the most conserved motifs), Positional bias (p-value) ≤ 0.001 (to select motifs whose positional distribution and/or distribution over both strands are less likely to be random). The information provided by MEME-LaB for each motif was then used to select the most significant motifs the following parameters for each motif in each group was

Table 5-10: Different FC cut-offs used for motif analysis in MEME-LaB (Brown et al., 2013).

Datasets	FC Cutoff
Dataset 1	FC > 3 or FC < -3
Dataset 2	FC > 3
Dataset 3	FC < -3
Dataset 4	FC > 2 or FC < -2
Dataset 5	FC > 2
Dataset 6	FC < -2
Dataset 7	All over / under expressed differentially expressed genes
Dataset 8	All over expressed genes
Dataset 9	All under expressed genes

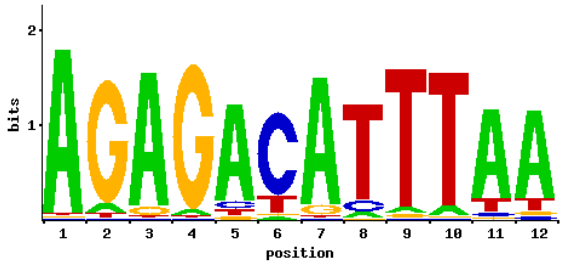
Figure 5-12

Motif 1



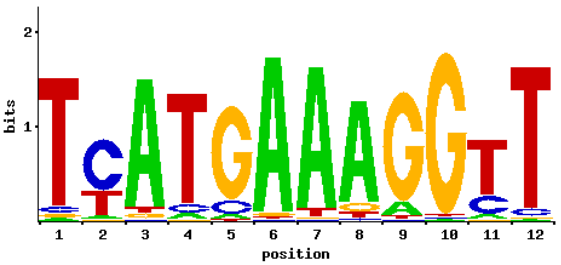
- Rhizobia effect at low N in A17 and *sunn-1* (groups 5 and 6)
- Rhizobia effect at high N in A17 and *sunn-1* (groups 7 and 8)

Motif 2



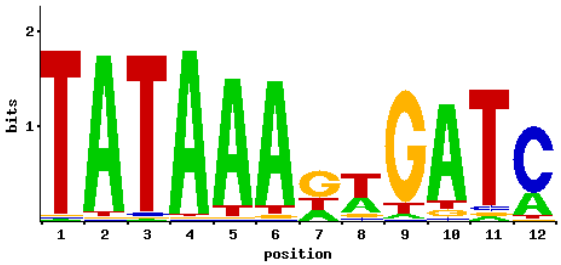
- Rhizobia effect at low N in A17 and *sunn-1* (groups 5 and 6)
- Rhizobia effect at high N in A17 (group 7)

Motif 3



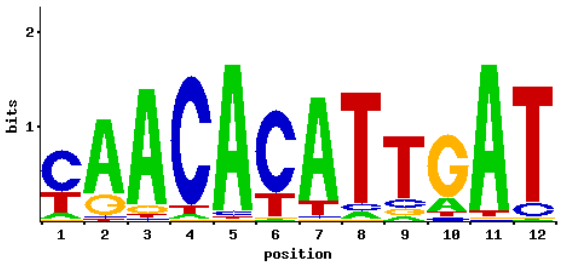
- Rhizobia effect at low N in A17 and *sunn-1* (groups 5 and 6)
- Rhizobia effect at high N in A17 (group 7)

Motif 4



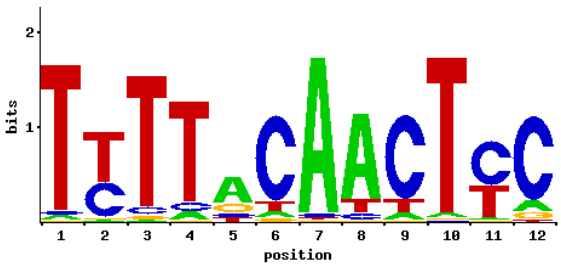
- Rhizobia effect at high N in A17 (groups 7)

Motif 5



- Rhizobia effect at high N in A17 (groups 7)

Motif 6



- Rhizobia effect at high N in A17 (groups 7)

Figure 5-12: Significant motifs shown as their motif logos discovered by MEME-LaB (Brown et al., 2013) and the groups of co-expressed genes they were found in. Groups correspond to the groups specified in table 5-3.

compared with other motifs of the group and also the same motif in other datasets: 1) having a higher number and percentage of promoter sites within the group in which it was found compared to other motifs in the same group; 2) larger total information content value (as number of bits) that is indicative of a larger or more conserved motif; 3) smaller E-value, indicating that the motif was unlikely to be a random artifact ; 4) positional bias expressed as *p*-value, that is the observed distribution of the motif compared to the length of the promoter sequences with an expected uniform distribution, lower *p*-value is indicative of a non-random distribution.

To identify biologically relevant findings, for each motif the top five closest matching known motifs and their PSSMs (position-specific scoring matrix) available in the JASPAR (Sandelin et al., 2004) and PLACE (Higo et al., 1999) public databases were analysed in MEME-LaB. The level of similarity to these known motifs was indicated as a distance score that compares the motif found by MEME to its closest matches among known motifs (smaller distance score values is indicative of a higher similarity). Motifs with close similarity to known motifs from *planta* were analysed in more detail. Since we were interested in studying the genes with strong (FC>2 or FC<-2) response to the N, rhizobia and *SUNN*, if any of these motifs were *only* discovered in the datasets containing all the differentially expressed genes with no FC cutoff, they were omitted from the selected list of motifs.

Based on all the above parameters six motifs were selected as significant motifs (Figure 5-12) among the 9 different datasets. These motifs were identified in the group of differentially expressed genes that were rhizobia-regulated at low N in A17 (group 5: motifs 1, 2 and 3) and *sunn-1* (group 6: motifs 1, 2 and 3); and rhizobia-regulated at high N in A17 (group 7: motifs 1, 2, 3, 4, 5 and 6) and *sunn-1* (group 8: motif 1). Some of the main features of the known motifs in *planta* that had the closest similarity to these motifs were nodulin, leghemoglobin and auxin response (Table 5-11). Around 70% of the promoters of the motifs were NCRs except for motif 1 that 30% of the promoters were NCRs. Aside from NCRs and proteins with unknown

Table 5-11: Closest matching known motifs in *planta* for the significant motifs discovered by MEME-LaB (Brown et al., 2013).

Motif	Closest matching known motifs		
	Natural name	**Species**	**Most interesting feature**
Motif 1	OSE2ROOTNODULE	*Medicago truncatula* *Glycine max (soybean)* *Vicia faba* *Sesbania rostrata*	Nodulin, Leghemoglobin
	NODCON2GM	*Glycine max (soybean)*	Nodulin, Leghemoglobin
	ARF Q2	*Arabidopsis thaliana*	Auxin response element (AuxRE)
	ARFAT	*Arabidopsis thaliana* *Glycine max (soybean)* *Oryza sativa (rice)*	Auxin; AuxRE; ARF; Aux/IAA
	GT1GMSCAM4	*Glycine max (soybean)*	Pathogen- and salt-induced
	SEBFCONSSTPR10A	*Solanum tuberosum (potato)*	Pathogenesis
	PYRIMIDINEBOXHVEPB	*Hordeum vulgare (barley)*	ABA, GA
Motif 2	OSE2ROOTNODULE	*Medicago truncatula* *Glycine max (soybean)* *Vicia faba* *Sesbania rostrata*	Nodulin, Leghemoglobin
	NODCON2GM	*Glycine max (soybean)*	Nodulin, Leghemoglobin
	ARFAT	*Arabidopsis thaliana* *Glycine max (soybean)* *Oryza sativa (rice)*	Auxin; AuxRE; ARF; Aux/IAA
	SURECOREATSULTR11	*Arabidopsis thaliana*	Sulphate, ARF
	PYRIMIDINEBOXHVEPB	*Hordeum vulgare (barley)*	ABA, GA
	PBF Q2	*Zea mays (maize)* *Hordeum vulgare (barly)* *Triticum aestivum (wheat)*	GA
	PYRIMIDINEBOXOSRAMY1A	*Oryza sativa (rice)* *Hordeum vulgare (barly)*	Sugar
	DOFCOREZM	*Zea mays (maize)*	Carbon metabolism

Table 5-11, continue

Motif	Closest matching known motifs		
	Natural name	**Species**	**Most interesting feature**
Motif 3	PBF Q2	*Zea mays (maize)* *Hordeum vulgare (barly)* *Triticum aestivum (wheat)*	GA
	PYRIMIDINEBOXOSRAMY1A	*Oryza sativa (rice)* *Hordeum vulgare (barly)*	Sugar
Motif 4	TATABOX2	*pea (Pisum sativum)* *tobacco (Nicotiana tabacum)* *bean (Phaseolus OS vulgaris)*	TATA; legA; phaseolin
	TAAAGSTKST1	*Solanum tuberosum (potato)*	KST1; Dof; guard cell
	NTBBF1ARROLB	*Agrobacterium rhizogenes*	auxin induction
	POLASIG1	*pea (Pisum sativum)* *rice (Oryza sativa)* *Arabidopsis thaliana*	poly A signal; NUE; FUE
Motif 5	RAV1AAT	*Arabidopsis thaliana*	RAV1; AP2; VP1; B3
Motif 6	CAREOSREP1	*Oryza sativa (rice)*	GA
Motif 7	OSE2ROOTNODULE	*Medicago truncatula* *Glycine max (soybean)* *Vicia faba* *Sesbania rostrata*	Nodulin, Leghemoglobin
	NODCON2GM	*Glycine max (soybean)*	Nodulin, Leghemoglobin
	ARF Q2	*Arabidopsis thaliana*	Auxin response element (AuxRE)
	ARFAT	*Arabidopsis thaliana* *Glycine max (soybean)* *Oryza sativa (rice)*	Auxin; AuxRE; ARF; Aux/IAA

Table 5-12: Putative promoters for the discovered significant motifs. Aside from NCRs and the proteins with unknown functions all the promoter motifs are shown in the tables.

Motif	Gene ID	Annotation (Mt3.5v5)
Motif 1	Medtr5g063670	Annexin
	Medtr8g038220	Annexin-like protein RJ4
	Medtr8g087710	Aquaporin NIP1-2
	Medtr8g022710	Cytochrome P450
	Medtr7g065770	Early nodulin ENOD18
	Medtr4g130800	Early nodulin-20
	Medtr4g093840	Flavonol synthase/flavanone 3-hydroxylase
	Medtr8g089360	High affinity cationic amino acid transporter 1
	Medtr6g044700	Late nodulin
	Medtr6g044570	Late nodulin
	Medtr5g066070	Leghemoglobin
	Medtr5g080400	Leghemoglobin
	Medtr5g080440	Leghemoglobin
	Medtr1g011540	Leghemoglobin
	Medtr5g081030	Leghemoglobin
	Medtr5g041610	Leghemoglobin K
	Medtr7g110180	Leghemoglobin K
	Medtr7g086040	MtN20 protein
	Medtr7g114890	MtN26 protein
	Medtr7g114880	MtN26 protein
	Medtr6g089330	MtN28 protein
	Medtr5g036110	MtN9 protein
	Medtr7g093010	Myb family transcription factor APL
	Medtr5g012270	Nitrate transporter
	Medtr5g084020	Nodule-specific glycine-rich protein
	Medtr5g084080	Nodule-specific glycine-rich protein 1H
	Medtr5g084040	Nodule-specific glycine-rich protein 1I
	Medtr2g042510	Nodule-specific glycine-rich protein 2B
	Medtr2g042470	Nodule-specific glycine-rich protein 2D
	Medtr3g055440	Nodulin 25
	Medtr4g081200	Non-race specific disease resistance protein 1-like protein b
	Medtr7g092230	Oligopeptide transporter OPT family
	Medtr7g092250	Oligopeptide transporter OPT family
	Medtr4g130790	Pectinesterase
	Medtr2g033630	Pectinesterase inhibitor
	Medtr1g116930	Peptide transporter PTR1
	Medtr3g072300	Peptide transporter PTR3-A
	Medtr7g098220	Peptide transporter PTR3-A
	Medtr7g098160	Peptide transporter PTR3-A
	Medtr7g098150	Peptide transporter PTR3-A
	Medtr7g098090	Peptide transporter PTR3-B
	Medtr7g031470	Receptor protein kinase-like protein
	Medtr5g025850	Receptor-like protein kinase
	AC233574 1	Receptor-like protein kinase At3g21340
	Medtr6g006140	Solute carrier family 2, facilitated glucose transporter member 2
	Medtr2g102010	Solute carrier family 25
	Medtr6g006260	Sugar transporter family protein
	Medtr4g014280	Tir-nbs-lrr resistance protein
	Medtr6g092500	Transcripteion factor

Table 5-12, continue

Motif	Gene ID	Annotation (Mt3.5v5)
Motif 2	Medtr1g030270	Early nodulin
	Medtr7g065770	Early nodulin ENOD18
	Medtr6g089330	MtN28 protein
	Medtr3g069420	Peptide transporter PTR3-B
Motif 3	Medtr3g055520	Calmodulin-like protein 3
	Medtr2g015470	cDNA clone J023134O11 full insert sequence
	Medtr4g107930	Cysteine proteinase
	Medtr8g076190	Kinesin heavy chain DNA binding protein-like
	Medtr6g044570	Late nodulin
	Medtr5g042350	LGC1
	Medtr5g036110	MtN9 protein
	Medtr5g084210	Nodule-specific glycine-rich protein 1C
	Medtr5g084100	Nodule-specific glycine-rich protein 1G
	Medtr6g078140	Pathogen-inducible trypsin-inhibitor-like protein
	Medtr6g078120	Pathogen-inducible trypsin-inhibitor-like protein
	Medtr7g098160	Peptide transporter PTR3-A
	Medtr7g098150	Peptide transporter PTR3-A
	Medtr7g098090	Peptide transporter PTR3-B
	Medtr8g101550	Phospholipase A2 homolog 3
	Medtr5g022390	Thiosulfate sulfurtransferase
	Medtr7g080940	Uncharacterized UDP-glucosyltransferase At1g05670
Motif 4	Medtr4g072980	Auxin-induced SAUR-like protein
	Medtr3g055490	Calmodulin-like protein 4
	Medtr3g044270	Cysteine proteinase
	Medtr5g099060	Nodule inception protein
	Medtr2g065210	Protein FAR1-RELATED SEQUENCE 5
	Medtr7g082810	Protein TRANSPARENT TESTA 12
Motif 5	Medtr5g008040	12-oxophytodienoate reductase
	Medtr5g061410	F-box protein At4g22280
	Medtr6g044700	Late nodulin
	Medtr6g044570	Late nodulin
Motif 6	Medtr1g030270	Early nodulin
	Medtr5g041610	Leghemoglobin K
	Medtr6g078280	Miraculin
	Medtr7g114880	MtN26 protein
	Medtr5g084260	Nodule-specific glycine-rich protein 1L
	Medtr7g104360	Purple acid phosphatase 22

function, other target genes were mainly nodulins, leghemoglobins and calmodulin-like proteins (Table 5-12). This made up for around 90% of the promoter motifs to be involved in the nodulation pathway. These all suggests that these motifs could be the regulatory components involved in controlling gene expression responses to low or high N to regulate the number of nodules, based on the plant N status.

5.3 Conclusion

Phenotypic study of RSA changes in response to N and rhizobia in A17 and *sunn-1* showed that the response was different between these two genotypes. At low N the number of nodules was significantly (P<0.05) higher in *sunn-1* compared to A17. However, average of LR length was significantly (P<0.01) less in the nodulating *sunn-1* samples than A17 (Figure 5-4). This was because LR total length was significantly (P<0.05) less in *sunn-1* and there was not a significant difference in number of LRs between A17 and *sunn-1* (Figure 5-1 b). At the high N concentration that was inhibiting for nodulation, *sunn-1* had the ability to nodulate but the number of nodules was significantly (P<0.05) less than in low N. Average of LR length was significantly (P<0.01) less in *sunn-1* compared to A17 at high N but number of LRs was (P<0.01) higher (Figure 5-1 b).

To study the balance between nodule number and LR development (based on the differences observed in the RSA responses in our phenotyping studies), the effect of rhizobia and *SUNN*-regulated gene expression changes were compared between *sunn-1* and A17 with a focus on the responses at low N because of the enhanced lateral root-AON effects in *sunn-1* at low N. Studying differentially expressed significantly (FC>2 or FC<-2) induced or repressed genes showed that rhizobia-regulated responses were stronger in *sunn-1* compared to A17 and that this was independent of the N concentration (Table 5-4 and figure 5-8 a). The strongest (higher number of significantly differentially expressed (FC>2 or FC<-2) genes) *SUNN*-regulated responses were observed in the presence of rhizobia especially at low N (Table 5-4 and

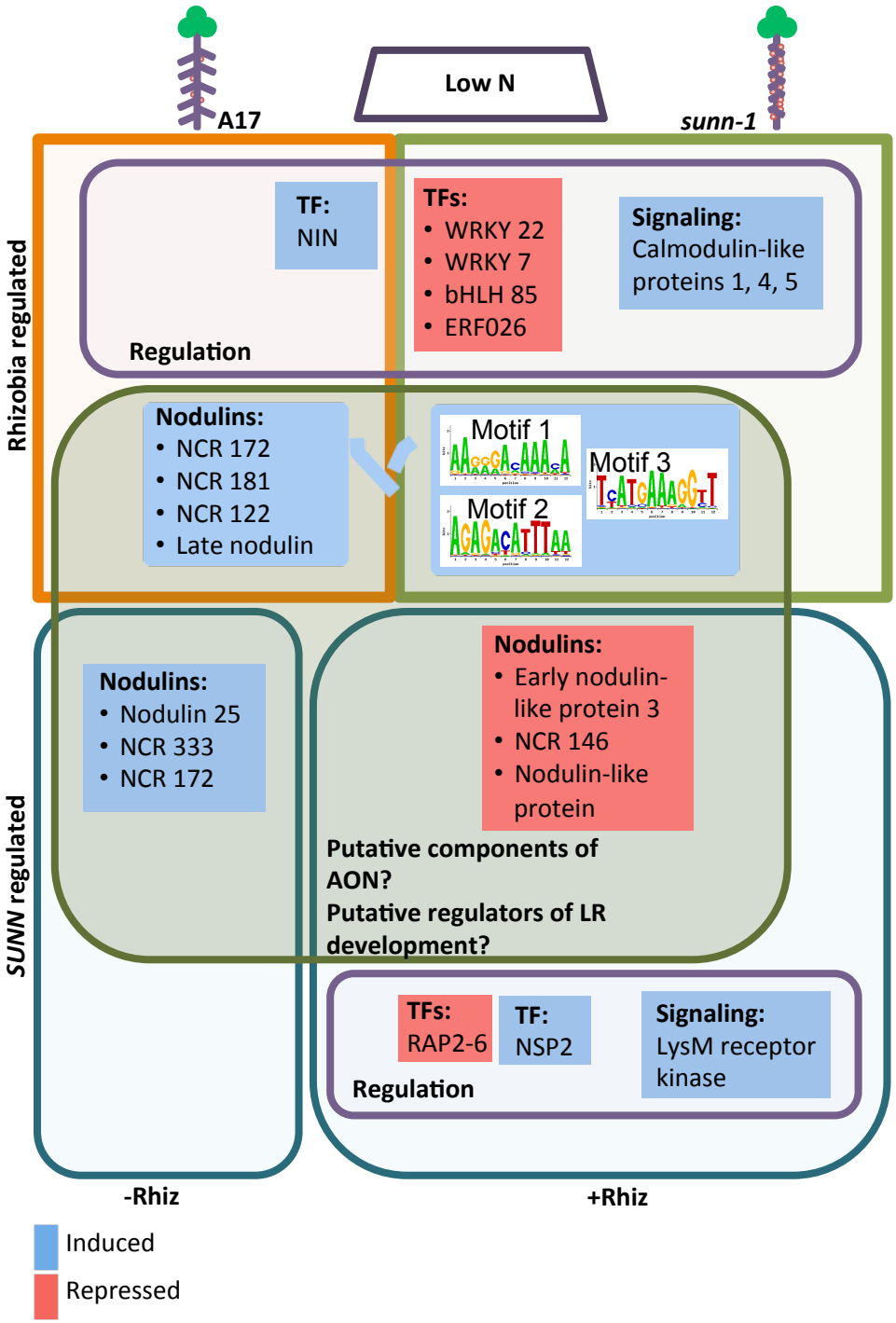
Figure 5-8 b). Based on the gene abundance of different MapMan biological functional categories (Figure 5-8 a and b) this proposes a model (Figure 5-9) suggesting that regulation (signaling, regulation of protein activity and TFs) and nodulins are the main factors responsible for the hypernodulation and short LR phenotype in *sunn-1* at low N.

The current study suggests that rhizobia-regulated and *SUNN*-regulated nodulins mainly from the NCR protein family, stress responses, TFs and other regulatory responses through signaling and regulation of protein activities at low and high N may be important in the *sunn-1* phenotype, and thus in AON control (Figure 5-13). We have identified nodulins with putative role in AON that may be also involved in the balance between LRs and nodules in low N (Table 5-6 and 5-7 a and b). These are different sets of differentially expressed nodulins with distinctive rhizobia-regulated and/or *SUNN*-regulated expression changes at low N (FC>2 or FC<-2), which correlate with phenotypic effects. Predominantly, rhizobia induced their expression at low N and they were more highly expressed in A17 compared to *sunn-1* (Figure 5-13 a). The *SUNN*-regulated effect on the expression of nodulins was rhizobia dependent, being *SUNN*-induced in the absence of rhizobia and *SUNN*-repressed in the presence of rhizobia.

A high percentage (43%) of the differentially expressed TFs (FC>2 or FC<-2) showed strong rhizobia-suppressed response (FC<-2) in *sunn-1* at low N. The higher number of rhizobia-regulated (FC>2 or FC<-2) differentially expressed TFs in *sunn-1* at low N that was three times more than A17 and that a high percentage were rhizobia-suppressed in *sunn-1*, could all suggest that these TFs are involved in the pathways leading to the control of nodule numbers and balance between LRs and nodules (Figure 5-13 a). Among these TFs, the number of WRKY TFs was higher than TFs from other families.

Based on the differences between gene expression patterns of A17 and *sunn-1* differentially expressed (FC>2 or FC<-2) rhizobia or *SUNN*-induced or repressed regulatory genes and nodulins at low N we have identified putative

Figure 5-13, (a)


A17
Low N
sunn-1
Rhizobia regulated
TF:
NIN
TFs:
• WRKY 22
• WRKY 7
• bHLH 85
• ERF026
Signaling:
Calmodulin-like proteins 1, 4, 5
Regulation
Nodulins:
• NCR 172
• NCR 181
• NCR 122
• Late nodulin
Motif 1
Motif 2
Motif 3
SUNN regulated
Nodulins:
• Nodulin 25
• NCR 333
• NCR 172
Nodulins:
• Early nodulin-like protein 3
• NCR 146
• Nodulin-like protein
Putative components of AON?
Putative regulators of LR development?
TFs:
RAP2-6
TF:
NSP2
Signaling:
LysM receptor kinase
Regulation
-Rhiz
+Rhiz
Induced
Repressed

(b)

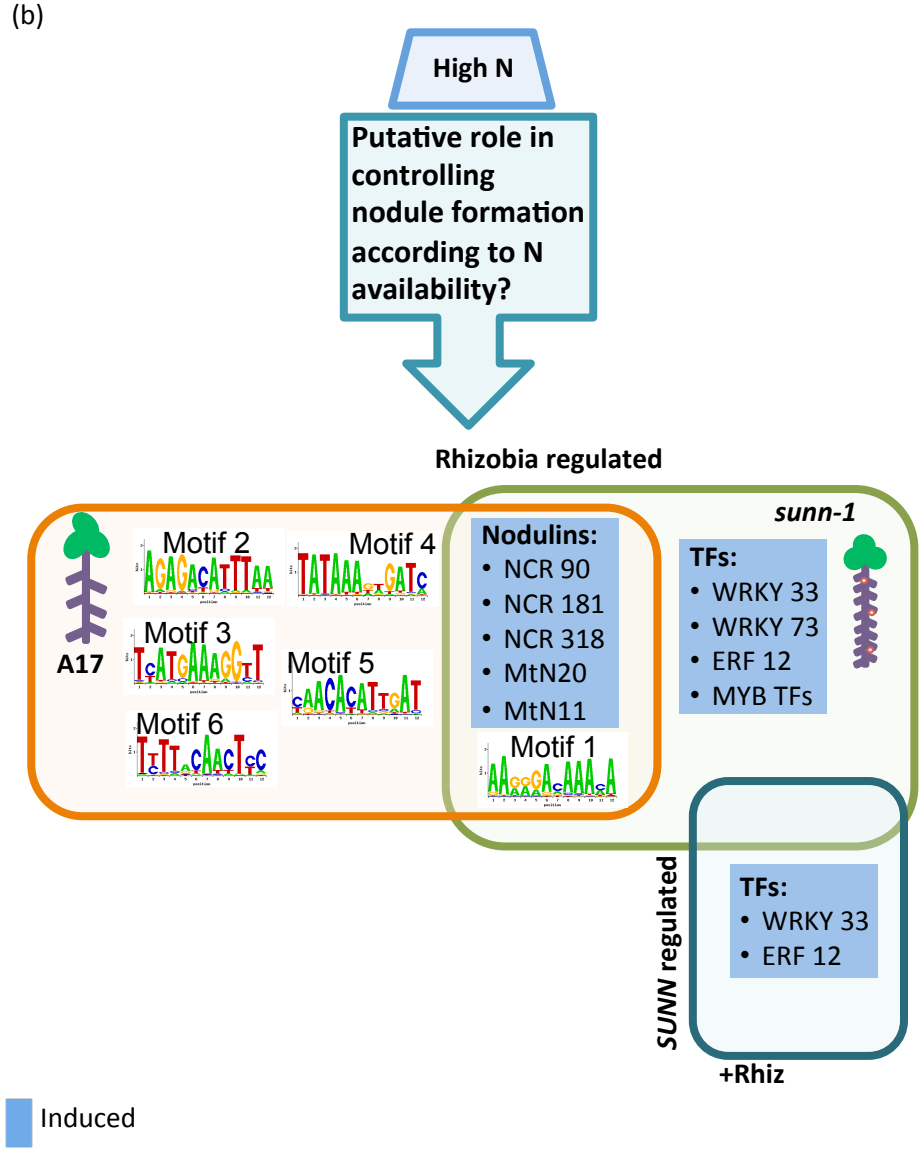


Figure 5-13: Candidate upregulated (blue) and downregulated (red) genes potentially involved in AON and the balance between nodule formation and LR development at (a) low and (b) high N. These are genes that were significantly (FC>2 or FC<-2) rhizobia-regulated in A17 or *sunn-1* (upper panels), or *SUNN*-regulated in the absence (-Rhiz) or presence (+Rhiz) of rhizobia (lower panel). The boxes pointing to the two green and orange lines in the middle indicate that the genes are expressed in both A17 and *sunn-1*.

roles in AON for some of the components of the nodulation pathway (Figure 5-11 and 5-13 a). Of the differentially expressed TFs regulating the expression of early and late nodulins during NF signaling and nodule organogenesis, the bifunctional TF NIN was found to be rhizobia-induced with a lower expression level only in *sunn-1* at low N. The ethylene responsive TFs ERF026 and RAP2-6 were rhizobia-repressed and *SUNN*-repressed respectively and the GRAS family TF NSP2 was *SUNN*-induced. The expression of this combination of TFs, signaling genes (e.g. LysM receptor kinase and calmodulin-like proteins 1, 4 and 5) and the differentially expressed (FC>2 or FC<-2) nodulins (Table 5-6 and 5-7 a and b) involved in the infection pathway (NF signaling pathway) and nodule organogenesis and the different expression patterns they show at low N in response to rhizobia and/or *SUNN* (in the presence of rhizobia) makes them suitable candidates for AON (Figure 5-13 a).

Studying the expression patterns of significantly (FC>2 or FC<-2) rhizobia and/or *SUNN*-induced or repressed genes at high N and comparing these responses between A17 and *sunn-1* and with their expression levels at low N enabled to candidate groups of TFs and nodulins as putative regulators of nodulation. The higher expression level of these rhizobia and/or *SUNN* regulated nodulins (e.g. NCR 90, MtN20 and MtN11) and TFs that function in developmental processes and response to biotic and abiotic stresses (e.g. WRKY 33 and ERF 12) in *sunn-1* compared to A17 at high N (Figure 5-13 b), could be controlling the ability of this mutant to nodulate at N levels otherwise inhibitory for A17. This could suggest that these specific nodulins and TFs may be involved in controlling nodule formation according to N availability.

Using motif analysis tools, six motifs were discovered: motif 1 (AAGGGACAACA), motif 2 (AGAGACAT), motif 3 (TCATGAAA), motif 4 (TATAA), motif 5 (CAACACA) and motif 6 (TTTTAC). The putative promoters of these motifs were mainly nodulins and genes involved in the nodulation pathway with the majority of the promoters being NCRs. These promoters were strongly (FC>2 or FC<-2) rhizobia regulated at low and/or high N

(Figures 5-13 a and b) and they could be regulators of gene expression responses in response to low and high N to control the nodule numbers.

As reported in this chapter, by combining our phenotyping results with gene expression studies on roots of A17 and *sunn-1* at low and high N in the presence or absence of rhizobia, we were able to propose models (Figures 5-11 and 5-13) for explaining the *sunn-1* phenotype and identifying some of the components of the nodulation pathway as regulators of LR development and nodule formation. As expected, some of the CLE peptides putatively involved in AON were not significantly differentially expressed in our study. This could be due to the choice of mutant (discussed in chapter 6) or a higher threshold could be selected for identifying the differentially expressed genes. The data provided in this study could be useful in identifying new N-regulated nodulins and *SUNN*-regulated genes that could be components of different biological pathways involved in balancing the plant N uptake with it's growth and development.

Chapter 6

General conclusion

Nitrate as a signal molecule regulates plant gene expression, metabolism, growth and development (Ruffel et al., 2008, Vidal and Gutiérrez, 2008, Bouguyon et al., 2012) for instance, by influencing hormone balances (Bouguyon et al., 2012, Wang et al., 2012), local stimulation of LR growth (Zhang and Forde, 1998, Walch-Liu et al., 2006, Gan et al., 2012) and LRP initiation (Vidal et al., 2010). The response of RSA to N availability has been widely studied. Significant progress has been also made to understand the molecular nature of communication between individual pairs of plant-microbes. This study focused on how LR development and nodule formation were balance in *Medicago* according to the N availability. This was achieved by combining phenotypic analysis of N-regulation of *Medicago* RSA with whole genome expression profiling of N responses. This enabled generation of a list of candidate genes that control the responses and cross talk in *M. truncatula* roots to N and symbiosis. Analysis of the regulatory genes and processes enabled a better understanding of how legumes balance N uptake and use from LRs with N_2 fixation.

6.1 Phenotypic studies of the RSA response to low and high concentrations of N and rhizobia

Phenotypic measurements of the root architecture were used to study root architecture changes in *M. truncatula* in response to low and high concentrations of N and rhizobia inoculation. The regulatory gene networks

targeted by shoot-root N status signals in the plant and the functional responses of the N acquisition systems are determined by the N form (Ruffel et al., 2008, Jeudy et al., 2010). In our studies we showed that the ability to nodulate and the presence or absence of rhizobia could affect root development of *Medicago* plants grown under low or high N conditions in the form of NH_4NO_3. The response of the rhizobia inoculated and non-inoculated plants of *M. truncatula* cv. Jemalong A17, cv. Jemalong 2HA, ssp. *tribuloides*, Gaertner and var. longispina to low (0.5 and 1 mM) and high (2 and 10 mM) N followed a similar trend (Figure 3-2 and 3-3). This could suggest that the response is conserved over these different genetic backgrounds of *Medicago*. Hence *M. truncatula* var. Jemalong A17 (sequenced reference accession, denoted here as 'wild type') was used as the model plant to study RSA responses to low and high N concentrations and rhizobia.

Subjecting A17 to a range of low and high concentrations of N (0-20 mM) showed that nodulation was highest at low N concentrations (0.1 and 0.5 mM) and it was inhibited at high N starting from 5 M. The inhibition of nodulation at high concentrations of N (Streeter, 1985, Thimm et al., 2004) in which N fixation capacity of existing nodules is also affected (Cabeza et al., 2014) is a balancing mechanism in the nodules to avoid depletion of carbon resources when other sources of N is available (Silsbury et al., 1986, Wery et al., 1986, Carroll et al., 1990).

In *M. truncatula*, N is acquired through three main pathways: NH_4, NO_3 and N_2 (Ruffel et al., 2008). Local and systemic pathways acting at the molecular level regulate NH_4 and NO_3 uptake and N_2 fixation in the nodules and root development depending on the N status of the whole plant (Remans et al., 2006a, Poultney et al., 2007, Desnos, 2008, Ruffel et al., 2008, Forde, 2014). Symbiotic organisms (Oláh et al., 2005, Maillet et al., 2011) and components of the legume symbiosis pathway such as flavonoids that are secreted from the plants as signal molecules or Nod factors that are signal molecules released by the rhizobia can also effect LR development (Oláh et al., 2005, Laffont et al., 2010). The data obtained from studying the response

of A17 root architecture to low (0.1 mM) and high (5 mM) concentrations of N showed that rhizobia had no significant (P<0.05) effect on root development (average of LR length, LR total length, LR number and LR density) at low N (Figure 3-5 e). Studying the time scale of LR development at low N also confirmed these results showing that rhizobia had no significant (P<0.05) effect on different stages (LRP i, LRP ii, emerging and fully emerged LRs) of LR development (Table 3-3). At low N, treated samples at the earlier time points in both -Rhiz and +Rhiz samples showed an increase in LR development (LRP i, emerging and fully emerged LR) with a decreasing trend at the later time points (Figure 3-9 a). In +Rhiz, as the number of nodules increased the number of LRs at different developmental stages (LRP i, emerging and fully emerged LR) decreased (Figure 3-9 a). This balancing response between LR number and the number of nodules that form and regulate N_2 fixation within the nodules could be to avoid excessive depletion of carbon reserves and to conserve energy pool in order to develop more nodules rather than produce more LRs.

To further study the effect of rhizobia and nodulation on different stages of LR development, -Rhiz plants grown at low N were inoculated with rhizobia and LR developmental changes were studied at 0, 4, 8, 12 and 16-day time points. The results from this study also suggested there was a balancing mechanism between LR and nodule formation. This was concluded because at early time points post-inoculation with rhizobia, the number of LRP i in +Rhiz was significantly (P<0.05) less compared to -Rhiz (Figure 3-9 a). Also in +Rhiz samples there was an increasing trend in the number of LRs at different developmental stages at the earlier time points but as the number of nodule primordia and mature nodules increased at the later time points, there was a decreasing trend in the number of LRs at different stages of development (Figure 3-9 c).

We have found that the presence of rhizobia, at high N concentrations that inhibit nodulation significantly (P<0.05) affected root development and had an inhibitory effect on PR length, LR total length, LR number and root

total size (Figure 3-5). This suggests tight coregulation of LR and nodule formation, with connection of gene regulatory pathways a likely mechanism underlying this. To see how high N concentration and rhizobia affected LR development, different stages of LR development was studied 0-16 days post treatment of -Rhiz and +Rhiz plants grown at low N with high N. The results from this study also showed that rhizobia significantly (P<0.05) affected early and late stages of LR development (LRP i and fully emerged LRs) (Table 3-3) at high N. It also showed that at early time points post treatment of N deprived plants with high N, there was an increasing trend in the number LRs in different stages of development (LRP i and ii, emerging and fully emerged LR) at the earlier time points that was followed by a decrease at the later time points (Figure 3-9 b). This response shows that the plant N status balances LR development.

6.2 Whole genome profiling of root responses to high N and rhizobia

Phenotypic studies showed that high N affected PR and LR development in the presence of rhizobia. In order to identify genes that control the plant's root response to N and rhizobia, whole-genome profiling was carried out. The phenotypic data was used to inform choice of the most suitable concentrations of N (0.1 and 5 mM) for the design of these transcriptomic experiments. Microarray experiments were used to study gene expression changes in whole root samples of rhizobia-inoculated or mock-inoculated A17 plants grown at low N at 0, 2 and 6 hours post-treatment with high N (Figure 4-2 a).

A total of 4793 genes were identified as differentially expressed with a significance cutoff of P<0.05 that represented 7.4% of the genes annotated in the *M. truncatula* 3.5 gene model. Differentially expressed genes with FC>2 or FC<-2 were identified as the genes showing the strongest expression changes under the experimental conditions. The number of these differentially expressed genes (FC>2 or FC <-2) showed that high N effect was stronger in -Rhiz at both 2 and 6 h and a higher number of genes were N-regulated

compared to +Rhiz. This suggested that the early (2 and 6 h) responses to high N were mainly independent of the rhizobia effect and N-induction especially at 2 h time point was the predominant response (Table 4-1).

A model (Figure 4-11) was proposed for the early (2 and 6 h) responses to high N in -Rhiz and +Rhiz N-deprived plants. This model identifies the differentially expressed genes with strong (FC>2 FC<-2) N (Figure 4-11 a) or rhizobia (Figure 4-11 b) regulated expression changes and the main biological functional categories they belong to. These genes could be responsible for the root phenotype observed at high in -Rhiz and +Rhiz (chapter 3 and Figure 4-1) balancing PR and LR development with nodule formation.

The results show that PNR genes (Figure 4-11 a) such as genes involved in N transport, assimilation and amino acid metabolism were rapidly (within 2-6 h) induced upon high N treatment (Figure 4-11 a). Studies in *Arabidopsis* also shows that PNR genes are significantly expressed shortly after treatment with nitrate in roots (Krouk et al., 2010b). Among these, NRT1.1 was strongly induced (FC=2.5) only in the presence of rhizobia. NRT1.1 is involved in auxin transport and accumulation which is required for LR growth (Bouguyon et al., 2012). The strong induction (FC=2.5) of NRT1.1 in response to high N only at +Rhiz could indicate that it may be involved in the regulatory response affecting the PR and LR length at high N in rhizobia-inoculated plants (Figure 3-5) and that its expression is affected by the presence of rhizobia. The PNR response to high N treatment was more enhanced in -Rhiz N-deprived plants possibly to compensate for the N deficiency they were facing. This could be suggested because of the stronger induction of ferredoxin-nitrite reductase (Table 4-4) that is involved in N assimilation (Orea et al., 2001) and the higher number of differentially expressed (FC>2 or FC<-2) amino acid metabolism genes in -Rhiz.

In response to high N treatment the circadian clock-associated protein 1a (Medtr8g077420.1) was strongly N-suppressed (Figure 4-11 a). This suppression was related to the duration of high N treatment (suggested by the

two times decrease in FC at 6 h compared to 2 h in -Rhiz and +Rhiz) and independent of rhizobia. The N-suppression of this TF was one of the strongest (FC<-4) between the N-suppressed differentially expressed genes at 6 h in both -Rhiz and +Rhiz. CCA1 with nitrate is involved in regulating some of the steps in N metabolism (Gutiérrez et al., 2008) and also the activity of NRT1.1 and NRT2.1 (Krouk et al., 2010a). The enhanced N-assimilation activities suggested by the strong upregulation of the differentially expressed high affinity nitrate transporters (NRT1.1 and NRT2.1) and nitrate reductases and the strong N-suppression (FC<-2) of CCA1 could be indicative of the regulatory response and interaction between the circadian rhythm and N-assimilation.

Enhanced developmental activities were also observed early (2 and 6 h) after treatment with high N. Some of the differentially expressed TCA cycle enzymes and proteins involved in carbohydrate metabolism specifically TCA carbonic anhydrases (Medtr5g066060) were strongly induced (FC>2) in -Rhiz and +Rhiz samples. In *Arabidopsis* carbon assimilation enzymes involved in N/carbon balance are regulated by nitrate (Sakakibara et al., 1997, Scheible et al., 1997). Here the strong induction of the TCA carbonic anhydrases (Figure 4-11) may be to provide the necessary carbon and energy pool for the enhanced metabolic and developmental activities due to N availability.

There was a predominant upregulation of significantly expressed genes related to the nodulation pathway showing very strong expression changes (2 < FC < 9) in response to rhizobia at low N (0 h) and high N (2 and 6 h). This could be indicative of an enhanced developmental activity in the roots under low and high N condition. In this study also 6 significantly expressed NCR peptides were identified that could have a putative role in the nodulation pathway. This is hypothesised because they were strongly rhizobia-induced (FC>2) only at low N (Table 4-6 b and Figure 4-11 b). The strong rhizobia-induction (FC>2) of another group of significantly expressed NCRs only at high N (Table 4-6 c and d, Figure 4-11 b) makes them putative regulators/inhibitors of nodule development at high N or candidates for the

rhizobia mediated LR development observed at high N condition (Figure 4-11).

The rhizobia-mediated response of N-sufficient plants to low N was also studied. Gene expression changes of whole root samples of +Rhiz plants grown at high N were studied 6 h post treatment with low N and a model was proposed for this response (Figure 4-12). The differentially expressed genes (FC>2 or FC<-2) in this study were predominantly (83%) N-suppressed. This could be indicative of the activation of a local N signal to decrease biological activities in response to low N condition. The strong (FC>2) induction of NRT2.1 by low N could suggest that it is involved in the regulation of this early response to low N. The strong suppression (FC<-2) of nitrate reductase also suggests the decrease in N assimilation at low N.

6.3 Identifying components of the AON with putative role in balancing LR development and nodule number

The phenotypic study of RSA changes in response to N and rhizobia showed that the response was different between A17 and *sunn-1*. This response was specially more enhanced in *sunn-1* at low N with a significantly (P<0.05) higher number of nodules and shorter LRs (average of LR length significantly (P<0.01) less due to significantly (P<0.05) smaller LR total length) than A17 (Figure 5-1). *sunn-1* is impaired in correct AON responses for regulating the number of nodules (Van Noorden et al., 2006). Previous studies suggest that AON genes could be candidates for regulating LR development in response to N (Jin et al., 2012). The hypernodulating phenotype of *sunn-1* with short LRs at low N suggests that the components of the AON pathway may also be involved in balancing LR development. To study this balance between LR development and nodule number whole genome expression profiling was used and the effect of rhizobia and *SUNN* regulated gene expression was compared between *sunn-1* and A17.

In this study 7186 genes (15% of the genes annotated in the *M. truncatula* 3.5 gene model) showed significant expression changes under the

experimental condition (Figure 5-5 a). The number of differentially expressed genes with significant (FC>2 or FC<-2) rhizobia and/or *SUNN*-regulated responses (Table 5-4) showed that irrespective of the N concentration, rhizobia-regulated responses were stronger in *sunn-1* compared to A17 (Table 5-4 and Figure 5-8 a) and the *SUNN*-regulated response was stronger in the presence of rhizobia especially at low N (Table 5-4 and Figure 5-8 b).

Nodulins made up for the majority of the differentially expressed (FC>2 or FC<-2) rhizobia-regulated genes at both low and high N in A17 and *sunn-1* (Figure 5-8 a). We have identified different sets of differentially expressed nodulins with distinctive rhizobia-regulated and/or *SUNN*-regulated expression changes at low N (FC>2 or FC<-2) that may be involved in the balance between LRs and nodules in low N with a putative role in AON (Table 5-6 and 5-7 a and b). This is suggested because the expression changes in these nodulins in response to rhizobia and/or *SUNN* correlated to the root phenotype at low N (Figure 5-13 a). The hypernodulating phenotype with short LRs at low N in *sunn-1* was rhizobia dependent and different from A17. Differentially expressed (FC>2) rhizobia-induced nodulins at low N had a higher expression level in A17 compared to *sunn-1*. Also, the expression changes of differentially expressed (FC>2 or FC<-2) nodulins affected by *SUNN* were rhizobia-dependent. They were *SUNN*-induced in the absence of rhizobia and *SUNN*-repressed in the presence of rhizobia.

The expression patterns of the differentially expressed TFs at low N also correlated to the difference between *sunn-1* and A17 root phenotype at low N in +Rhiz. The number of rhizobia-regulated (FC>2 or FC<-2) differentially expressed TFs in *sunn-1* at low N was three times higher than A17 of which around 43% were rhizobia-suppressed in *sunn-1.* Among the *SUNN*-regulated differentially expressed TFs at low N, significant (FC>2 or FC<-2) expression changes were only observed in the presence of rhizobia (Figure 5-8 b and 5-10 b). It is possible that this high rhizobia-suppressed response of TFs in *sunn-1* at low N and the *SUNN*-regulated response that is significant (FC>2 or

FC<-2) only in the presence of rhizobia may be related to the absence of control in the number of nodules in *sunn-1* (Figure 5-13 a).

Some of the differentially expressed (FC>2 or FC<-2) known components of the nodulation pathway that were rhizobia or *SUNN*-regulated showed differences in gene expression patterns between A17 and *sunn-1* at low N. Because of these differences in expression patterns and taking into account the differences between root phenotype of A17 and *sunn-1* at low N (Figure 5-3) we propose that they may have putative roles in AON (Figure 5-11 and 5-13 a). LysM receptor kinase (Medtr5g086130.1) was differentially expressed at low N and *SUNN*-induced in the presence of rhizobia. This receptor is involved in the perception of the Nod factor (Amor et al., 2003). Calmodulin-like proteins 1, 4 and 5 (Medtr3g055570.1, Medtr3g055490.1, Medtr3g055510.1 respectively) were rhizobia-induced (2.3<FC<6.8) but their expression levels were almost two times lower in *sunn-1* than A17 at low N (Table 5-8). These proteins are involved in calcium spiking that leads to root hair curling and activation of DMI3 (Oldroyd, 2013). NIN TF (Medtr4g068000.1) that is involved in regulating the expression of early and late nodulins during NF signaling and nodule organogenesis (Andriankaja et al., 2007, Marsh et al., 2007, Yoro et al., 2014) was rhizobia-induced with a lower expression level only in *sunn-1* at low N. Of other TFs involved in the nodulation pathway ERF026 (Medtr1g101550.1) was rhizobia-repressed at low N in *sunn-1* and RAP2-6 (Medtr3g098580.1) was *SUNN*-repressed at low N in the presence of rhizobia. ERF026 and RAP2-6 are members of the ERF TF family. Members of this family are key regulator in the nodulation pathway and control of nodule number and differentiation (Andriankaja et al., 2007, Vernié et al., 2008). NSP2 (Medtr3g072710.1) that with NSP1 is a positive regulator of ERN1 and ENOD11 transcription (Cerri et al., 2012) was *SUNN*-induced at low N in the presence of rhizobia (Figure 5-11).

The differentially expressed genes with significant (FC>2 or FC<-2) rhizobia or *SUNN* regulated responses were also studied using motif analysis tools (Brown et al., 2013). From these studies, six motifs were discovered:

motif 1 (AAGGGACAACA), motif 2 (AGAGACAT), motif 3 (TCATGAAA), motif 4 (TATAA), motif 5 (CAACACA) and motif 6 (TTTTAC). Because these promoters were strongly (FC>2 or FC<-2) rhizobia-regulated at low and/or high N (Figures 5-13 a and b) and their putative promoters were mainly nodulins and genes involved in the nodulation pathway (the majority being NCRs) we suggest that they may be regulators of nodule numbers depending on the plant N status.

6.4 Future work

Phenotyping and transcriptomic analysis were used to study the effect of rhizobia and N on the balance between LR and nodule development. We studied the time scale of LR and nodule development in response to rhizobia and N at cellular level. The time scale of early stages of nodulation in response to low and high N could be identified more precisely by visualising the GFP-carrying *S. meliloti* strain using confocal microscopy (Gage et al., 1996, Fournier et al., 2015).

In this study we were able to identify some of the key pathways and genes that were involved in the balance between LR and nodule development according to the N availability in *M. truncatula*. Whole genome expression profiling of N and rhizobia responses in roots enabled us to generate list of candidate genes and identify biological pathways involved in regulating root and nodule responses to rhizobia and high N. By comparing the responses of A17 and *sunn-1* to N (low and high) and rhizobia we have also identified genes with putative roles in AON and the balance between nodule number and LR development. The data generated from this work could be integrated with other available transcriptomic data (such as Ruffel et al., 2008). This could give a comprehensive perspective on the genes involved in the root and nodulation responses in *Medicago* and would enable comparisn between different data sets. The nodulation genes in cluster 26 (section 4.2.3.4) could be interesting in this regard and it can give new insight into unknown nodulation genes.

The pathways and genes indicated in the hypothesised models (Figures 4-11, 4-12, 5-11 and 5-13) could be key and interesting candidates to further study the balance between N uptake and use from LRs with N_2 fixation in the nodules. The expression of these differentially expressed genes (FC>2 or FC<-2) could be validated under different conditions (e.g. different time points, N and rhizobia treatment) and with different known and available mutants using q-PCR. In this study also 6 motifs were identified with putative promoters mainly involved in the nodulation pathway. These could be validated using yeast two-hybrid systems.

The choice of rhizobial symbiont could affect the efficiency of nodules in N_2 fixation (Mhadhbi et al., 2005, Terpolilli et al., 2008). Plant N acquisition is also strongly affected by the choice of bacterial partner (Laguerre et al., 2012). In this study, the reference strain *S. meliloti* RCR 2011 was used as *M. truncatula* symbiont. It has been reported that this strain is poorly effective in N_2-fixation with Mt Jemalong A17 (Mhadhbi et al., 2005). A more N-efficient strain such as *Sinorhizobium medicae* strain md4 that shows to have a higher symbiotic performance (Laguerre et al., 2012) could be used to study the balance between LR and nodule development based on N availability. Comparing these two transcriptomic data could then give us a better understanding of the effect of low and high performance symbionts and N on the balance between LR and nodule in the plant to meet its N demand.

The cross talk between LR and nodule development are highly regulated. The role of hormones is crucial in this cross talk and regulation. In our study we have identified genes involved in different hormone signaling pathways such as auxin, JA, SA and GA that showed significant (FC>2 or FC<-2) expression changes in response to N (Tables 4-4 and 4-5). These hormones have well known roles in control of LR and nodule number. SA, JA and GA are involved in regulating LR development through cross talk with auxin pathway. SA with auxin regulates PIN trafficking and auxin flux and distribution (Armengot et al., 2014, Du et al., 2013). JA negatively regulates auxin transport by affecting the distribution of PIN family auxin transporters in

the plasma membrane (Sun et al., 2011, Sun et al., 2009). GA is a negative regulator of LR development by affecting the polar auxin transport (Gou et al., 2010). JA and SA are also involved in regulation of nodulation. There are different reports on the negative and positive roles of JA in regulating nodule number (Nagata and Suzuki, 2014). SA is a strong inhibitor of nodulation (van Spronsen et al., 2003, Stacey et al., 2006) with a direct role in signal transmission in the autoregulation of nodulation (van Spronsen et al., 2003, Sato et al., 2002). The genes we have identified in these pathways were differentially (FC>2 or FC<-2) expressed in response to N. These genes could be involved in integrating JA, SA or GA signalling into auxin pathway to cross-regulate LR and nodule development in response to N. These data could be used in studying N regulation in these pathways, how it could affect root and LR development and whether there is a direct connection between these pathways in response to N.

To study the AON role of *SUNN*, other alleles rather than *sunn-1* could be a better choice (Schnabel et al., 2010). For example *sunn-4* that is a putative null mutant of *SUNN*. Schnabel et al. (2005) had identified four alleles of *SUNN*. The *sunn-1* mutation that is most often used by researchers is resulted from an amino acid change in the kinase domain of the protein in a residue highly conserved in serine/threonine and tyrosine kinases (Schnabel et al., 2010, Hanks and Quinn, 1991). The *sunn-4* allele creates a stop codon, which should result in truncation of the protein immediately after the initial signal peptide sequence and is presumed to act as a null mutation (Schnabel et al., 2005, Schnabel et al., 2010). The fact that most of the CLE peptides (Mortier et al., 2010) with putative role in AON were not significantly (FC>2 or FC<-2) differentially expressed in our study in *sunn-1* could be related to our choice of mutant. Using other alleles such as *sunn-4* we could study the N-regulated expression of CLE and CEP peptides. The effect of *SUNN* on controlling nodule number and LR development could be then studied. Split root or grafting experiments (Ruffel et al., 2008, Jeudy et al., 2010) could be used to investigate if this *SUNN* effect on root architecture is a local or systemic response.

Finally, this study could continue at a cell-specific level. Pericycle and cortical cell responses to rhizobia and N could be studied over a time period (e.g. a 48 hour time course). Generating stable *M. truncatula* transgenic lines expressing GFP in pericycle and cortex was not successful in our study. Although the early generations expressed strong and cell specific GFP, this expression was silenced in the later generations. Using hairy root transformation (Deng et al., 2011) could be an alternative way to express the cell type specific GFP that could be used for cell sorting. Using cell type profiling and systems biology approaches it would be then possible to generate models for the root developmental interactions with the environment.

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